Tissue-specific expression of the human type II collagen gene in mice

(embryonic stem cells/chimeras/transgenic mice/DNA-mediated gene transfer)

ROBIN H. LOVELL-BADGE*[†], ANNE BYGRAVE*, ALLAN BRADLEY[‡], ELIZABETH ROBERTSON[‡], RITA TILLY[§], AND KATHRYN S. E. CHEAH[¶]

*Medical Research Council Mammalian Development Unit, Wolfson House, 4 Stephenson Way, London NW1 2HE, United Kingdom; [‡]Department of Genetics, University of Cambridge, Cambridge CB2 3EH, United Kingdom; [§]Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom; and [¶]Biochemistry Department, University of Hong Kong, Sassoon Road, Hong Kong

ABSTRACT   Type II collagen is crucial to the development of form in vertebrates as it is the major protein of cartilage. To study the factors regulating its expression we introduced a cosmid containing the human type II collagen gene, including 4.5 kilobases of 5' and 2.2 kilobases of 3' flanking DNA, into embryonic stem cells *in vitro*. The transformed cells contribute to all tissues in chimeric mice allowing the expression of the exogenous gene to be studied *in vivo*. Human type II collagen mRNA is restricted to tissues showing transcription from the endogenous gene and human type II collagen is found in extracellular matrix surrounding chondrocytes in cartilage. The results indicate that the cis-acting requirements for correct temporal and spatial regulation of the gene are contained within the introduced DNA.

Type II collagen is unique among the abundant collagens in showing a high degree of tissue specificity. It is found essentially only in cartilage where it is the major and characteristic extracellular component. It is made primarily by chondrocytes that arise as condensations of mesenchymal cells in an inductive response to overlying ectoderm (1, 2). In the embryo, the correct regulation of type II collagen is important not only for those structures that remain as cartilage but also for bone formation because hypertrophic cartilage forms the framework onto which the bone matrix is deposited during endochondrial ossification (3, 4). This occurs progressively in the fetus, and a general switch from type II to type I collagen is seen, as bone-forming cells functionally replace chondrocytes as producers of the extracellular matrix (5). However, the production of type II collagen by chondrocytes in the epiphyseal plate is important for the continuing growth of long bones, and a number of structures, such as the sternum, remain as cartilage into adult life.

An understanding of the mechanisms involved in the differentiation of cartilage is of key importance not only to the study of development but also in prospects for cartilage and bone replacement therapy. Cartilage degradation is a feature of arthritis and it is also likely that defects in the type II collagen gene are associated with some of the inherited growth disorders in humans that affect cartilage or bone development (6, 7).

To study the factors regulating type II collagen gene expression we have introduced a cosmid containing the human type II collagen gene (8), including 4.5 kilobases (kb) of 5' and 2.2 kb of 3' flanking DNA (Fig. 1) into embryonic stem (ES) cells *in vitro* (9, 10). By taking advantage of the ability of ES cells to contribute to chimeric mice after injection into host blastocysts (11, 12), we hoped then to be able to study the expression of the gene *in vivo*. Preliminary data had suggested that this approach was indeed feasible (13), and this is confirmed by the much more extensive analysis reported here. We present evidence for the tissue-, cell-, and stage-specific expression of the human type II collagen gene in the chimeras. The human gene is shown to be correctly transcribed, and human type II collagen is synthesized in chimeric mouse cartilage.

MATERIALS AND METHODS

DNA-Mediated Gene Transfer. The cell lines and calcium phosphate/DNA precipitation technique for ES cells have been described elsewhere (12–14).

Chimeras. Host blastocysts (3.5-day) from MF1 strain albino mice were injected with ≈12 ES cells (11, 12). Chimeras were recognized initially by the presence of pigment in coat and/or eyes, and donor cell contribution was estimated by glucose-6-phosphate isomerase isozyme and Southern blot analysis.

Southern Blotting. *Eco*RI-digested DNA samples (10 μg) were electrophoresed on 0.7% agarose gels and transferred to nitrocellulose. The probe used was the 7.3-kb *Eco*RI fragment as in cosHcol.1 (Fig. 1). A high-stringency wash (15 mM NaCl/1.5 mM sodium citrate, pH 7/0.5% NaDodSO$_4$, 65°C) was used in Fig. 2 *A* and *B*, whereas Fig. 2*C* was at lower stringency (75 mM NaCl/7.5 mM sodium citrate, pH 7).

RNase Protection Assays. Total cellular RNA was prepared by 6 M urea/3 M LiCl differential precipitation (13, 14). RNase protection assays were carried out as described (14, 15). pS4.EP.9 linearized with *Eco*RI and pS5-PH1.2 linearized with *Pst* I were used to generate ^{32}P-labeled complementary RNA to the 3' and 5' ends, respectively, of human α1(II) mRNA. Each hybridization reaction used 7 × 10^5 dpm of probe. Numbers of copies of human and mouse α1(II) mRNA were estimated either by determining dpm in bands cut out of the gels or by densitometry, relating the signal in sample tracks to known amounts of transcripts run on the same gels. The two largest major protected fragments (about 80 and 70 bases) were each used to determine levels of endogenous mouse transcription, and both gave similar results. In chimeric tissues, the percentage of ES cell contribution is taken into account, and the proportion of chondrocytes in a tissue sample can be determined by comparing signal intensities with those given by pure cartilage samples of the same age.

Immunolabeling. Human material was obtained from fresh vacuum aspirates. Control mouse embryos were from normal matings of MF$_1$ strain mice, and chimeric embryos were recognized from their littermates by the presence of eye

Abbreviations: HFC, human fetal cartilage; ES, embryonic stem.
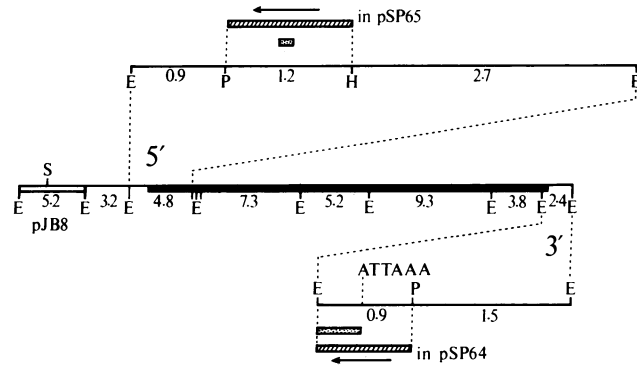[†]To whom reprint requests should be addressed.

FIG. 1. Restriction map of cosHcol.1 and derivation of subclones. Human α1(II) coding sequence is represented by the filled box and is about 30 kb, although the mature mRNA is only 5.9 kb. EcoRI restriction sites (E) and sizes of the EcoRI fragments are indicated. For transformation, cosHcol.1 was linearized at the unique Sal I site (S) within the vector. The 3' probe: the 900-base-pair (bp) EcoRI/Pst I fragment, which covers the end of the transcribed sequence from 35 bases upstream of the termination codon to the poly(A) addition site, was ligated into pSP64, to give pS4-EP.9. The 5' probe: the 1200-bp Pst I/HincII fragment from the 4.8-kb EcoRI subclone was cloned into pSP65, to give pS5-PH1.2. This 1200-bp fragment contains the transcription start site (unpublished observations). Hatched boxes represent fragments subcloned into SP6 vectors, and stippled boxes show the portions of these that cover transcribed sequences.

pigmentation. The embryos in Fig. 4 *C* and *D* were chimeras showing >30% Gpi-1^a in liver and tail biopsies and considerable pigmentation in both eyes. That in Fig. 4 *E* and *F* had a contribution of only 5% from CN1. Embryos were washed in phosphate-buffered saline (PBS), fixed in fresh 1% monomeric gluteraldehyde in 0.1 M phosphate (pH 7.3) at 4°C for 1.5 hr, washed, and stored at 4°C in phosphate buffer. The tissues were embedded in polyethylene glycol, sectioned at 2–4 μm, and applied to acid-washed glass microscope slides. Sections treated with bovine testicular hyaluronidase (Sigma, 4000 units/ml) to remove glycosaminoglycan were washed extensively in PBS containing 0.15% Tween 20 and 10% normal goat serum and incubated overnight at 4°C in dilutions of monoclonal antibody to human type II collagen in the same buffer (16). The slides were extensively washed and incubated for 1 hr in second antibody: 5-nm gold-conjugated goat anti-mouse IgG (Janssen Pharmaceutica, Beerse, Belgium). Silver enhancement was carried out with the IntenSE kit from Janssen.

RESULTS

DNA-Mediated Gene Transfer into ES Cells and Chimera Formation. cosHcol.1 and pSV$_2$neo were used to cotransform the ES cell line EK-HD14 by the calcium phosphate/ DNA precipitation method. The transformation rates in two separate experiments were 2.55 and 2.01 × 10^{-6} G418-resistant colonies per cell per μg of pSV$_2$neo. Approximately 70% of transformants contained the human gene with copy number varying from 1 to about 100 (Fig. 2A).

Three transformed sublines were used to derive chimeras: CN1 and CN15, each containing from 5 to 10 copies, and CN8, containing about 100 copies of cosHcol.1 per diploid genome. The overall frequency of chimera formation was 47%, which compares favorably to that found for the original untransformed cell line. CN1 and CN15 each gave up to 50% contribution within individual chimeras, with a fairly even distribution to all tissues analyzed (Fig. 2C). CN8, however, showed a very uneven distribution, with contributions ranging from 0 to 20% in various tissues within an animal (Fig. 2B). This was also shown by the pattern of pigmentation,
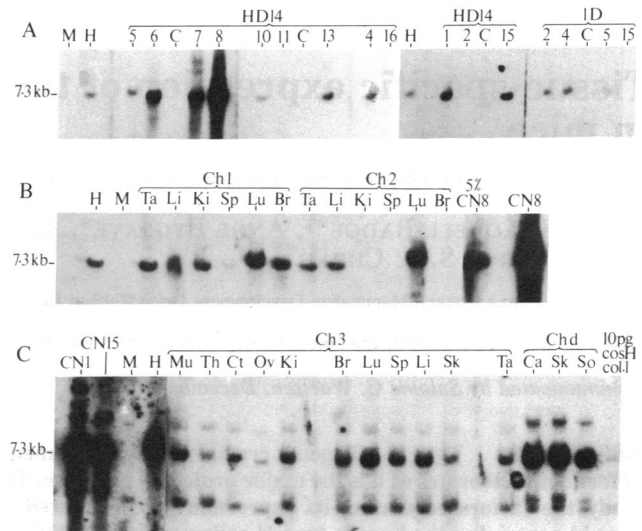

FIG. 2. Southern gel analysis to detect cosHcol.1 in DNA from cotransformed cell lines and chimeric mouse tissues. (A) Analysis of G418-resistant cells. M, control mouse liver; H, human placenta; C, control untransformed cells. The numbers refer to clones of transformed HD14 ES cells and Clone 1D fibroblasts. (B) Analysis of tissues dissected from two 4-week-old chimeric mice derived with the HD14 transformant CN8. Chimera 1 (Ch1) showed significantly more coat pigmentation than chimera 2 (Ch2). Ta, tail; Li, liver; Ki, kidney; Sp, spleen; Lu, lung; Br, brain. (C) Analysis of tissues dissected from an 8-week adult and an 18.5-day embryonic chimera derived with CN1 and CN15, respectively. Endogenous bands are present due to the use of a lower stringency wash. Chimera 3 (Ch3) ovary and chimera d (Chd) soft tissue samples are underloaded with respect to the other tissues. Mu, muscle; Th, thymus; Ct, cartilage; Ov, ovary; Sk, skin; Ca, carcass; So, soft tissues (viscera and brain).

chimeras from CN1 and CN15 being much more "fine grained" than those from CN8. This unevenness cannot be due to a simple restriction in potential, as CN8 seems able to contribute to all cell types. All three sublines show an X:14 translocation, which existed in ≈30% of the original untransformed HD14 cell population. Otherwise they have a normal karyotype (data not shown). No morphological abnormalities that could be attributed to the donor cells were seen in over 150 newborn and 30 fetal chimeras.

RNase Protection Assays to Detect α1(II) mRNA in Control Tissues. When [^{32}P]UTP-labeled RNA transcripts from the plasmid pS4-EP.9 (3' probe) (Fig. 1) are hybridized to total RNA from 14-week human fetal cartilage (HFC), fragments of 430 bases corresponding to the 3' end of human type II collagen mRNA [α1(II) mRNA] are protected from digestion by RNase (Fig. 3A). The size of the protected fragment indicates that neither of the consensus poly(A) signals (AATAAA) at 195 and 1389 bases from the stop codon (197) is being used (17). Possible alternative sites could be an ATTAAA sequence at 415 bases or ATTTTTAAA at 395 bases (18, 19). The chicken type II collagen poly(A) signal is known to be at position 415 (20).

With transcripts from pS5-PH1.2 (5' probe), protection assays with HFC RNA reveal a major band of 140 bases, together with a much less intense band at 240 bases (Fig. 3B). The former corresponds to the first exon, whereas the larger fragment represents unspliced precursor or transcripts from an alternative start site. Bands of equivalent sizes are not seen with either probe when total RNA from mouse fetal sternal cartilage is used, demonstrating the specificity of these RNase protection assays. However, a number of smaller bands are seen that correspond to short regions of homology between the mouse and human type II collagen genes. These are useful as indicators of endogenous mouse

FIG. 3. RNase protection assays for human type II collagen gene transcripts. 3'T, 3' transcript; 5'T, 5' transcript; NRC, no RNA control; Ri, ribs; MFS, control mouse fetal sternum; MF, 16.5-day control mouse fetus. See Fig. 2 legend for additional abbreviations. (A) Assay with the 3' probe for human α1(II) mRNA in cell lines, in tissues from a 12-week adult chimera CN15.12 (about 30% stem cell origin), and from 16.5-(b) and 18.5-day postcoitum (d and e) chimeric embryos. Twenty micrograms of total RNA was assayed in each track except for calvaria (Cv), 7 µg; control mouse fetal sternum (MFS), 1 µg; and HFC, 1 µg. Numbers refer to bases. (B) Assay with the 5' probe to look for correct initiation from cosHcol.1 in cell lines and chimeric embryos. Twenty micrograms was assayed in each track except for Cv, 7 µg; MFS, 2 µg; and HFC, 2 µg. Embryo a was derived with CN1, and embryo e was derived with CN15.

gene transcription and help define appropriate tissue-specific expression.

When RNase protection assays were carried out with total RNA from a stable Cl-1D fibroblast transformant, 1DCN4, a band of 413 bases, was clearly seen with the 3' probe. No such band was found with RNA from control, untransformed, Cl-1D cells (Fig. 3A). The level of human α1(II) mRNA in 1DCN4 is considerably lower than in HFC; we estimate from a number of 3' protection studies that the former has about 40 such mRNA molecules per cell, compared with about 2000 for the latter. No bands representing transcripts from the endogenous mouse α1(II) gene are seen, indicating that expression of the human gene is inappropriate in 1DCN4. With the 5' probe, there are a number of large protected fragments (e.g., at 350 bases) in the 1DCN4 track (Fig. 3B) that are not seen with HFC RNA. These could represent readthrough from upstream promoter elements (in flanking DNA, cotransfected pSV2neo, or cryptic sites within cosHcol.1). However, there is also a band at 140 bases, suggesting that at least a proportion of transcripts are correctly initiated.

cosHcol.1 Transcription in Undifferentiated Stem Cells. RNase protection assays using the 3' probe were performed on total RNA from the three transformed ES sublines used to derive chimeras, CN1, CN15, and CN8, from four other cotransformants, and from control untransformed HD14 cells (Fig. 3A). Human type II collagen mRNA can be detected in all of the transformed sublines except one, CN4. No bands representing endogenous transcripts can be seen, so this expression has to be considered as inappropriate. However, we estimate that the number of molecules of human α1(II) mRNA per cell is extremely low—e.g., 1 per cell for CN15 and 0.25 for CN1. The highest level is found in CN8, with 5–10 molecules per cell. This subline has a significantly greater number of copies of integrated cosHcol.1 (>100) than the rest, but otherwise there is no correlation between the

level of expression and number of integrated copies. Protection assays using the 5' probe with RNA from CN1 and CN15 (Fig. 3B) show some correct initiation, although CN1 gives a pattern of bands similar to 1DCN4 with the large protected fragment of 350 bases.

cosHcol.1 Transcription in Chimeras. To determine the pattern of expression from cosHcol.1, a number of chimeras were examined in detail for the presence of human α1(II) mRNA. The results obtained with CN1 and CN15 have been almost identical, so for simplicity we present mainly those for CN15. We have not looked for expression in chimeras derived with CN8 because the uneven distribution of donor cells would have necessitated studying large numbers of animals.

Representative results of RNase protection assays using the 3' probe with RNA from embryonic, newborn, and adult chimeras are shown in Fig. 3. Significant levels of human α1(II) mRNA, as revealed by the diagnostic band at 430 bases, are present in all tissues or embryo portions expected to contain chondrocytes—i.e., ribs, sternum, or carcass samples from embryonic and newborn chimeras. These tissues also show the smaller protected fragments—e.g., those at 80 bases—characteristic of mouse α1(II) mRNA. Two other tissues also express mouse and human α1(II) mRNA. These are the eye, which shows very low levels, and calvaria. Interestingly, 18.5-day calvaria shows a comparatively higher level of human transcript than mouse. We have been unable to detect human α1(II) mRNA in any adult chimera tissue, although a low level of endogenous transcript can be seen in cartilage.

Conversely, there was no evidence of any transcription of the exogenous gene in tissues that do not express mouse α1(II) mRNA. Estimates of the extent of chimerism were obtained from eye or coat pigmentation, from glucose-6-phosphate isomerase isozyme analysis, and, in some cases, from Southern blots. These verify that transcription was not

occurring in tissues even with a high contribution from the transformed stem cells. For example, embryo d was about 30% chimeric in all three portions (Fig. 2C) but only expresses human and mouse α1(II) mRNA in the "carcass" sample (Fig. 3A).

The results on tissue distribution of human α1(II) transcripts are summarized in Table 1. We estimate that in 16.5- to 18.5-day embryos there are ≈2000 endogenous α1(II) transcripts per chondrocyte [similar to values determined for the chicken (2)]. By comparison, the number of human α1(II) transcripts per ES cell-derived chondrocyte is about 600 (30%) for CN15 and 400 (20%) for CN1. The levels of human and mouse transcripts show a parallel decline as development proceeds.

The 5' probe was also used in RNase protection assays to see if transcription of human α1(II) mRNA was initiated correctly in the chimeric embryos. Protected fragments of the same size as in the HFC track are clearly evident in those from the appropriate tissues of CN1 and CN15 chimeras (Fig. 3B). With CN1, there is a shift to a more "normal" pattern from that seen in the undifferentiated cells. Preliminary evidence suggests that pSV2neo is regulated independently from cosHcol.1; at least we have been unable to detect its expression in chimeras (13). If the abnormal initiation of α1(II) transcription is due to readthrough from adjacent neo genes, then their down-regulation may explain such a shift.

Human Type II Collagen in Chimeric Mice. A mouse monoclonal antibody specific to human type II collagen (16) was used to test for the protein in fixed tissue sections from 14.5-day chimeric embryos. Normal mouse embryos of the same age and portions of 10-week human embryos were analyzed in the same way to provide controls. Intense labeling was seen in the extracellular matrix surrounding chondrocytes in the human material (Fig. 4A), and species specificity of the first antibody was shown by the absence of label over cartilage in control mouse embryos (Fig. 4B). However, there was clear labeling of extracellular matrix in cartilage of chimeric embryos, from CN1 and CN15 (Fig. 4 C and D). Labeling was seen only in cartilage, in patches throughout the body as expected from the chimeric nature of the tissue. This latter point is illustrated clearly in Fig. 4 E and F, which show a section through the region of the orbit in a low-grade CN1 chimera. Pigment granules are seen in the eye, confirming that it is chimeric, and there is a small patch of antibody staining in the frontal bone.
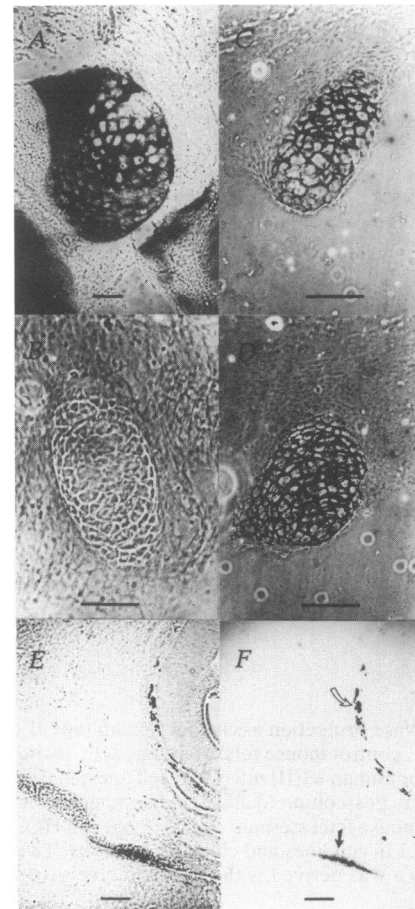

FIG. 4. Antibody localization of human type II collagen in tissue sections. (A) Human embryo, 10 weeks. (B) Control mouse embryo, 14.5 days. (C and D) CN15 chimeric embryos, 14.5 days: sectioned through the thorax and showing antibody staining specifically in the extracellular matrix surrounding chondrocytes in the ribs of the human and chimera samples. (E and F) Phase contrast (E) and brightfield (F) view of the same section through the head of a low-grade 14.5-day CN1 chimera. This section shows parts of an eye, including pigmented retina (open arrow), and a small patch of antibody staining in the frontal bone (solid arow). (Bars = 100 μm.)

Table 1. Human α1(II) transcripts in chimeric mouse tissues

	Age, days				
	14.5 pc	16.5 pc	18.5 pc	12 pp	>42 pp
Carcass	++++	++++	+++		
Soft tissues	−	−	−		
Sternum			+++	++	−
Ribs			+++	+	−
Calvaria			++	±	
Eyes			±	±	
Brain			−	−	−
Skin			−	−	−
Tongue			−		−
Lu, Th, Ki, Li, heart, Mu, Sp, blood				−	−

Estimates of relative abundance were obtained from 3' probe RNase protection assays. +++, Very abundant; ±, just detectable; −, not detectable; pc, postcoitum; pp, postpartum. See Fig. 2 legend for additional abbreviations.

DISCUSSION

Introduction of Foreign Genes into Mice by Means of ES Cells. Embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, have been used as recipients in gene transfer experiments to study the expression of a number of genes during differentiation in vitro (21–23) and in tumors (24). More recently, constitutive expression of neo genes, contained within retroviral vectors, has been observed in chimeras derived with EC and ES cells (25). We show here that it is possible to study the expression of a developmentally regulated gene in chimeric mice with the gene introduced into ES cells with a standard calcium phosphate/DNA precipitation technique. Moreover, the expression of the gene is regulated correctly in the chimeras.

The ES cell/chimera approach should complement the pronuclear injection method for introducing DNA into mice (26–28). It extends the range of possible experiments since it combines the advantages of an in vitro system with an in vivo one, allowing transformants to be characterized before introduction into an embryo or allowing a very rare event to be selected. It should be invaluable for introducing into mice constructs that are designed to be deleterious. The effects of these could be studied in chimeras or their offspring, as suggested by recent demonstrations of germ-line transmis-

sion of exogenous DNA sequences introduced *in vitro* (29, 30).

Expression of cosHcol.1. The data presented here show that the DNA sequences within 4.5 kb upstream and 2.2 kb downstream of the human type II collagen gene were able to mediate the expression of the gene correctly from mRNA to the final protein product. Human $\alpha1(II)$ mRNA transcripts were detected only in tissue samples that contained endogenous mouse $\alpha1(II)$ mRNA, and antibody staining revealed the human protein to be localized to the matrix surrounding chondrocytes. Both observations suggest that expression of the exogenous gene in the chimeras was tissue specific. The finding that correct regulation is conferred by sequences close to and/or within the transcription unit agrees with results obtained with a number of other genes (26–28). That it is a human gene being expressed in mouse suggests that regulatory mechanisms for type II collagen have not diverged widely.

There are a number of anomalies, however, that deserve some discussion. The low level of human $\alpha1(II)$ mRNA in undifferentiated ES cells is inappropriate. Selection for *neo* expression means that the site of integration is likely to be active. A nearby transcribing gene, or pSV$_2$*neo* itself, could be responsible for this expression, either because of an enhancer-like activity or because of readthrough transcription. Indeed, analysis of initiation of transcription in CN1 suggested some readthrough. This low-level expression is maintained during *in vitro* differentiation (results not shown), but, on reintroduction into a normal embryonic environment the regulation appears to become appropriately tissue specific.

The level of human $\alpha1(II)$ mRNA in chimeric tissues is generally less than the endogenous gene product. This could be due to the presence of vector sequences that can have an effect on expression in transgenic mice (27), although this may not be so critical here because of the selection for *neo*. On the other hand, fetal calvaria showed high levels of human $\alpha1(II)$ mRNA. We have no explanation for this at present, but it should be emphasized that mouse and human calvaria clearly show a low level of endogenous $\alpha1(II)$ transcription. Calvaria and the cosHcol.1 transformed fibroblast line 1DCN4 make $\alpha1(I)$ collagen (results not shown), which implies that the transcription of type I and type II collagen is not mutually exclusive as was previously thought.

We have not yet looked at chimeric embryos younger than 14.5 days postcoitum and so do not know whether high levels of human $\alpha1(II)$ collagen synthesis correlate with the first appearance of chondrocytes. However, in all other respects the pattern of expression of the human gene would appear to be as dependent on the developmental behavior of chondrocyte precursors as is the endogenous gene. For example, the human gene is transcribed in chondrocytes with diverse origins. Thus, it is clearly made by sternum and ribs, which are derived from lateral plate and segmented mesoderm, respectively (31). Also, antibody staining showed the protein to be present in the frontal bone (Fig. 4), which is thought to have its origins in the neural crest (32). In addition, the overall synthesis of human $\alpha1(II)$ mRNA decreases towards adulthood, reflecting the functional replacement of chondrocytes through their further differentiation to bone-forming cells. Chondrocytes cultured *in vitro* tend to "dedifferentiate," becoming fibroblastic, and show reduced levels of type II collagen (33, 34). This lability may reflect the original developmental option faced by mesenchymal cells (35) or the process of bone formation. The ability to direct expression of exogenous genes to chondrocytes may provide a means to

understand how factors such as cell shape, cell interactions, and matrix components can determine gene activity as well as allow us to define more precisely the cis sequences necessary for type II collagen expression.

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