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Introduction of the human proα1(I) collagen gene into proα1(I)-deficient Mov-13 mouse cells leads to formation of functional mouse–human hybrid type I collagen

(osteogenesis imperfecta/human–mouse collagen heterotrimer)

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ABSTRACT **The Mov-13 mouse strain carries a retroviral insertion in the proα1(I) collagen gene that prevents transcription of the gene. Cell lines derived from homozygous embryos do not express type I collagen although normal amounts of proα2 mRNA are synthesized. We have introduced genomic clones of either the human or mouse proα1(I) collagen gene into homozygous cell lines to assess whether the human or mouse proα1(I) chains can associate with the endogenous mouse proα2(I) chain to form stable type I collagen. The human gene under control of the simian virus 40 promoter was efficiently transcribed in the transfected cells. Protein analyses revealed that stable heterotrimers consisting of two human α1 chains and one mouse α2 chain were formed and that type I collagen was secreted by the transfected cells at normal rates. However, the electrophoretic migration of both α1(I) and α2(I) chains in the human–mouse hybrid molecules were retarded, compared to the α(I) chains in control mouse cells. Inhibition of the posttranslational hydroxylation of lysine and proline resulted in comigration of human and mouse α1 and α2 chains, suggesting that increased posttranslational modification caused the altered electrophoretic migration in the human–mouse hybrid molecules. Amino acid sequence differences between the mouse and human α chains may interfere with the normal rate of helix formation and increase the degree of posttranslational modifications similar to those observed in patients with lethal perinatal osteogenesis imperfecta. The Mov-13 mouse system should allow us to study the effect specific mutations introduced in transfected proα1(I) genes have on the synthesis, assembly, and function of collagen I.**

Type I collagen, a major structural protein of the extracellular matrix, is a heteropolymer that requires correct association of component polypeptide chains for its biological function. Functional type I collagen molecules are triple helical structures composed of two α1(I) and one α2(I) chains. These are synthesized as precursor proα1(I) and proα2(I) chains that undergo a series of co- and posttranslational processing steps (for review, see refs. 1 and 2). Some proline and lysine residues are hydroxylated and subsequently some of the hydroxylysine is further modified by glycosylation. The proα chains align, presumably following disulfide bond formation in their C-terminal propeptide domains, and triple helix formation occurs from the C terminus toward the N terminus. Following helix formation the procollagen molecule is secreted from the cell and the propeptide domains are proteolytically removed to produce functional collagen molecules, which undergo fibrillogenesis.

The protein sequence of collagen chains is highly conserved in evolution and only a 4% divergence between the α chains of species as distant as mouse and human has been found (3). This high evolutionary conservation suggests that the primary sequence of the polypeptide subunits is important for their biological function. That correct association of the chains in a triple helical molecule is crucial for the proper function of type I collagen is demonstrated in certain patients with lethal perinatal osteogenesis imperfecta (OI, type II). Mutations that affect the stability or alignment of the α chains such as a deletion (4, 5) or a point mutation (6, 7) may lead to a lethal disease. In these well-defined cases, and in others in which the exact molecular defect has yet to be determined, it is thought that the mutations disturb helix propagation, resulting in the commonly observed increase in the posttranslational hydroxylation and glycosylation of lysine (8, 9).

One experimental approach for studying the effect mutations may have on correct collagen assembly would be to introduce a gene that codes for one polypeptide component of collagen into cells that express the other component. For this, the endogenous gene corresponding to the transferred gene needs to be functionally deleted, with no effect on the expression of the other subunit components. In Mov-13 mice (10), transcription of the proα1(I) gene is completely blocked as a result of integration of the Moloney leukemia virus at the 5′ end of the gene (11). Transcription of the proα2(I) gene is not affected by viral integration (12). However, no functional α2(I) chains are detected in embryos by immunostaining (13) or are synthesized in cell lines from homozygous embryos (M.D., R. Timpl, and R.J., unpublished). This is probably due to rapid degradation of the proα2(I) procollagen chains, which are unable to form stable triple helices in the absence of proα1(I) chains (14). In this paper we show that transfer of either the mouse or the human proα1(I) gene into homozygous Mov-13 cells resulted in formation of stable type I collagen heterotrimers in association with the endogenous mouse proα2(1) chain.

MATERIALS AND METHODS

Plasmid Construction. Restriction enzymes, T4 DNA ligase, and *Sal* I linkers were purchased from Boehringer Mannheim and Bethesda Research Laboratories and were used under recommended conditions. The human proα1(I) collagen gene was derived by restriction digestion from the human cosmid clone CG103, whose structure has been

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Abbreviations: SV40, simian virus 40; OI, osteogenesis imperfecta.

described (15, 16). The mouse α1(I) collagen gene (clone 10D) was isolated from a murine cosmid library by screening with a human α1(I) cDNA clone (21). A map indicating the major restriction sites of both clones is depicted in Fig. 1. DNA clones containing different promoter fragments were constructed by subcloning an *Xba* I/*Eco*RI fragment, obtained from a partial *Xba* I and a complete *Eco*RI digest of either the mouse or the human cosmid clone, into the *Xba* I/*Eco*RI sites of pUC13 (see Fig. 1, *Xba* I site). Into the *Eco*RI site of these constructs a 5.6-kilobase (kb) *Eco*RI fragment for the human gene or a 5.6-kb fragment plus a 4.5-kb fragment for the mouse gene were cloned. This gave rise to a promoter minus genomic proα1(I) clone (20 kb) containing both poly(A) sites (for the 4.8-kb and 5.8-kb mRNA transcripts) and a single *Sal* I cloning site at the 5′ end of the gene in which the *Sal* I linkered promoter fragments were inserted.

Transfection of Mov-13 Cells. The origin of Mov-13 mice has been described (10, 12). Homozygous Mov-13 embryo cell lines were derived from embryos of heterozygous parents. Embryos were isolated at day 12 of gestation, and the cells were dispersed by treatment with trypsin and plated on tissue culture dishes. To derive permanent cell lines, the cultures were transformed with simian virus 40 (SV40). The genotype of the cell lines was determined by Southern blot analysis (11).

Genomic proα1(I) collagen clones were cotransfected with a selectable marker, pAG-60, which contains the neomycin phosphotransferase gene (17) at a molar ratio of 1:5, into the homozygous cell line M13 as described. After selection in 0.3 mg of G418 per ml, individual cell clones were isolated.

RNA Transfer Blot Analysis. Total cellular RNA was prepared according to the method of Auffray (18). After glyoxalation (19) the RNA was electrophoresed in a 1% (wt/vol) agarose gel, transferred to GeneScreen*Plus* (New England Nuclear), and hybridized to the end-labeled, human-specific, 43-base-pair (bp) synthetic oligonucleotide probe, which corresponds to nucleotides 180–222 of the transcribed region. This covers the 27-bp insertion in the first exon of the human gene and a few base pairs to either side (11, 16). RNA blots were also hybridized to the nick-translated α1(I) collagen cDNA probe Hf 677 (20, 21), the α2(I) collagen cDNA probe Hf 32 (22), and the rat α1 tubulin probe (23). Hybridization was also performed by using type IV collagen cDNA clone pPE 123, which was a kind gift from F. Ramirez.

Procollagen Labeling. All cell lines were set up for culture at equal cell densities and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum until 1–2 days after visual confluency. Collagen labeling and analysis were performed as described (9). The growth medium was replaced with fresh DMEM containing 10% (vol/vol) dialyzed fetal calf serum, 0.15 mM sodium ascorbate, and 0.1 mM β-aminopropionitrile fumarate. In some experiments, ascorbate was omitted and 0.1 mM α,α′-dipyridyl was included to prevent posttranslational lysine and proline hydroxylation. After a 4-hr preincubation, L-[5-^{3}H]proline (27 Ci/mmol, Amersham; 1 Ci = 37 GBq) was added (5 μCi/ml) and labeling was continued for 18 hr. The medium and cell layer fraction were analyzed separately. Procollagen and collagen were precipitated from the cell layer and medium fractions with 25% saturated $(NH_4)_2SO_4$ at 4°C. The precipitates were collected and redissolved in 0.05 M Tris·HCl, pH 7.5/0.15 M NaCl. Portions were prepared for electrophoretic analysis by digestion with 100 μg of pepsin per ml at 4°C for 16 hr, as described (9).

The extent of collagen proline hydroxylation was determined by dual-labeling with [4-^{3}H]- and [^{14}C]proline followed by specific bacterial collagenase digestion as described by Chojkier *et al.* (24).

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Samples were quantitatively loaded and electrophoresis was performed on 5% (wt/vol) polyacrylamide gels containing 2 M urea (9, 25). Radioactive gel bands were detected by fluorography (26). Collagenous proteins were identified by digestion of some samples with bacterial collagenase before gel electrophoresis (27). For quantitation, the bands were excised from the dried gel, hydrated with H_2O, and digested in 1 ml of Protosol (New England Nuclear) for 1 hr at 50°C. The released radioactivity was assayed by scintillation counting.

RESULTS

Transcription of the Mouse and Human proα1(I) Collagen Gene Transfected into Mov-13 Cell Lines. Various clones containing either the complete mouse or the human genomic proα1(I) collagen gene (Fig. 1) were cotransfected into the homozygous Mov-13 embryo cell line M13 (12) with a selectable marker (pAG-60) (17) that confers resistance to the neomycin analog G418. The three different mouse clones were clone 10D, which contains about 4 kb of sequences upstream of the AUG start of translation, and derivatives of this clone containing either 2.5 kb or 1.0 kb of 5′ flanking sequences. The human clones were CG103 (15), which contains about 1.6 kb of upstream sequences, and a derivative in which the human promoter was substituted for the SV40 early promoter/enhancer fragment (map position 5171–270) (28). After selection for G418 resistance, cell clones carrying the transfected collagen DNA were isolated. Southern blot analysis showed the presence of multiple collagen genes, some of which were rearranged (data not shown). For further analysis, cell clones were chosen that had at least one copy of the introduced gene integrated in an intact form.

RNA transfer blot analyses were performed to determine whether full-length proα1(I) collagen mRNA was expressed in the transfected cell lines. To show that human proα1(I) collagen mRNA was expressed in the Mov-13 cell lines, an end-labeled 43-bp synthetic oligonucleotide that covers the 27-bp insertion in the first exon of the human gene (for details, see *Materials and Methods*) was used as a probe. This probe was specific for the human gene, as no hybridization signal was seen in control mouse cells or in Mov-13 cells carrying the transfected mouse gene (Fig. 2*A*, lanes 9 and 11). Two cell lines (M13-SV-H1 and

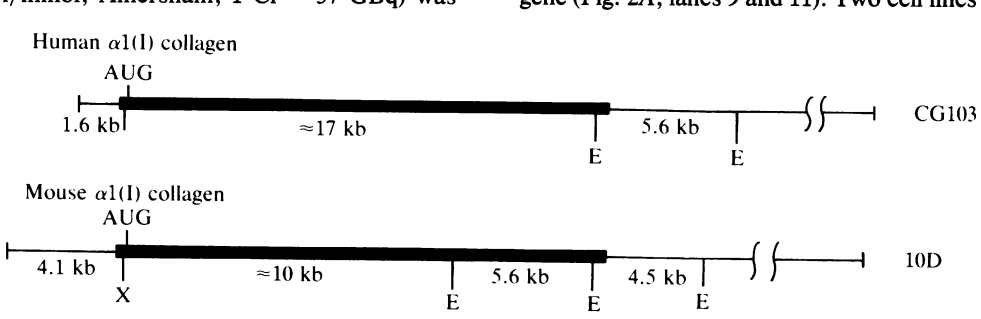

FIG. 1. Schematic map of the human α1(I) collagen cosmid clone (CG103) and the mouse α1(I) cosmid clone (10D). The black bar indicates the structural gene. Also shown are all of the *Eco*RI sites and the *Xba* I site adjacent to the translation start codon.

M13-SV-H4) containing the inserted human gene expressed both mRNA species of 4.8 kb and 5.8 kb (Fig. 2*A*, lanes 3 and 5), whereas another (M13-SV-H22) expressed only the smaller 4.8-kb mRNA (Fig. 2*A*, lanes 2 and 10). The presence of RNAs of less than full length was seen in some clones (Fig. 2*A*, lanes 2, 4, and 10). This is most likely due to transcription from aberrant copies of the transfected gene.

The same filter was rehybridized with the human α1(I) cDNA clone (Hf 677) (21), which detects equally well the human and mouse mRNA. In addition to the results obtained with the human-specific probe (Fig. 2*A*), Fig. 2*B* shows that the cell line M13-M5 selected from Mov-13 cells transfected with the mouse proα1(I) collagen gene expressed both of the correct mRNAs (Fig. 2*A*, lane 11). Similar to the results in Fig. 2*A*, shorter than full-length RNA species were detected with this probe in some samples. Between 20% and 50% of all analyzed G418-resistant cell clones expressed full-length proα1(I) collagen RNA from the transfected gene.

The level of collagen transcription was dependent on the promoter used. The transfected genes were transcribed at high levels when under the control of the SV40 promoter (Fig. 2, lanes 2–4 and 10) or under the control of upstream mouse sequences (lane 11). No difference was seen with mouse clones containing 2.5-kb or 1.0-kb upstream sequences (data not shown). In contrast, the human gene driven by its own promoter (1.5-kb upstream sequences) was expressed at much lower levels and was only detectable after overexposure of the RNA transfer blot (lane 1). This result was confirmed by S1 nuclease analysis (data not shown).

As expression of the proα1(I) and the proα2(I) genes is required for the formation of type I collagen, RNA from transfected cell lines was also hybridized to the human α2(I) cDNA clone (Hf 32) (22). Fig. 2*C* shows the presence of the 4.6-kb and 5.1-kb α2(I) RNA species at comparable levels in most cell lines. Differences in expression seem most readily explained by clonal variations. For example, differences in proα2(I) mRNA levels were seen between the uncloned M13 cell line, M13a, which represents a subpopulation of cells derived from the M13 cell line, and the cell clone M13-SV-H2 (Fig. 2*C*, lanes 4, 6, and 8).

To investigate whether different levels of exogenous proα1(I) or endogenous proα2(I) RNA expression were correlated with the expression of other endogenous collagen genes, the RNA transfer blot shown in Fig. 2*C* was rehybridized with the mouse α1(IV) cDNA clone pPE 123. The proα1(IV) mRNA of 6.8 kb was expressed in all cell lines. The amount of proα1(IV) mRNA showed the same clonal variation as the endogenous proα2(I) mRNA (data not shown). No correlation between the expression of the exogenous proα1(I) mRNA and other endogenous collagen genes was seen.

Synthesis of Procollagen in Transfected Cells. Synthesis of procollagen and the ability of the proα chains to form a triple helix was assessed after labeling cells with [^{3}H]proline and limited digestion of proteins with pepsin. Collagen molecules that have a stable helical conformation are resistant to limited pepsin digestion, which cleaves the N- and C-terminal propeptide regions. Collagen α1(I) and α2(I) chains were present in wild-type and in transfected homozygous cell lines after pepsin treatment (Fig. 3), demonstrating that helix formation had occurred between the endogenous proα2(I) chains and the proα1(I) chains synthesized from the inserted genes. Quantitation of the amount of pepsin-resistant collagen in the cell layer and medium fractions from each cell line showed that ≈50% of type I collagen molecules were secreted in all cell lines. This provided further evidence that the constituent α1(I) and α2(I) chains were assembled in a correct triple-helical conformation, since this is a prerequisite for collagen secretion. Type I collagen produced by the cell line containing the mouse insert was similar to wild-type collagen when analyzed by gel electrophoresis (Fig. 3).

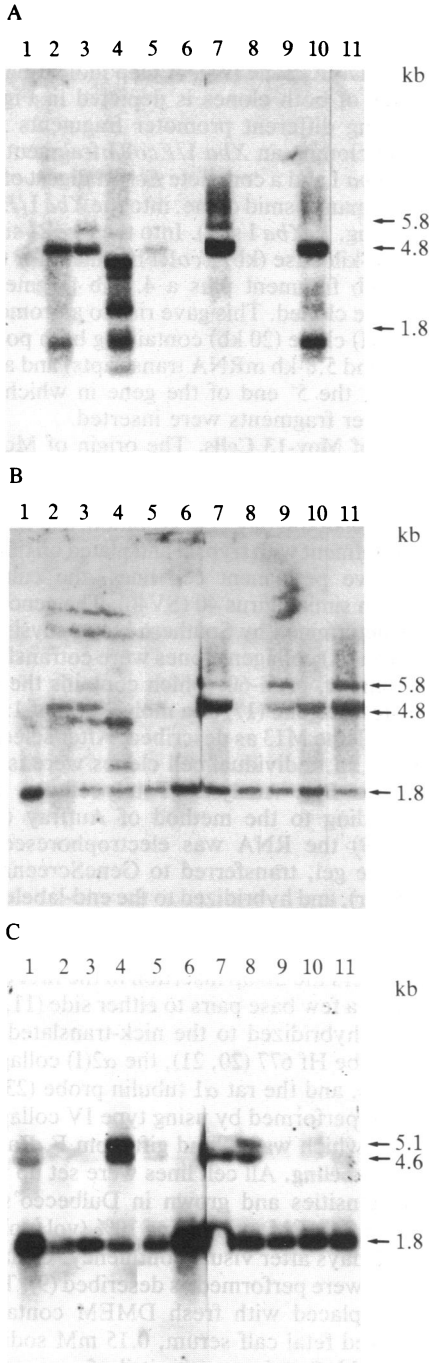

FIG. 2. Expression of full-length human or mouse collagen RNA. (*A*) RNA from cell clones transfected with the human gene or the mouse gene was hybridized to the human-specific 43-bp synthetic oligonucleotide probe (see Fig. 1). Lane 1, total RNA from a Mov-13 cell line (M13-H1) transfected with the human gene (CG103); lanes 2, 3, 4, and 5, RNA from M13-SV-H22, M13-SV-H1, M13-SV-H2, and M13-SV-H4, respectively, which were transfected with the human gene under SV40 control; lane 6, RNA from the homozygous Mov-13 cell line M13; lane 7, RNA from a human embryo fibroblast cell line (SV 80); lane 8, RNA from a subclone of M13 (M13a); lane 9, RNA from wild-type cell line; lane 10, RNA from an independent RNA preparation from M13-SV-H22; and lane 11, RNA from a cell line (M13-M5) transfected with the mouse gene (10D). The sizes of the two α1(I) mRNA transcripts (4.8 kb and 5.8 kb) are indicated. (*B*) RNA transfer blot from *A* rehybridized to the α1(I) cDNA clone Hf 677 (21). The rat α1 tubulin cDNA probe (23), which detects a 1.8-kb mRNA, was used as an internal standard for quantitative comparison. (*C*) RNA transfer blot of transfected Mov-13 cell lines hybridized to the α2(I) cDNA clone Hf 32 (22) and the rat α1 tubulin cDNA probe. RNA samples were loaded in the same order as in *A*.

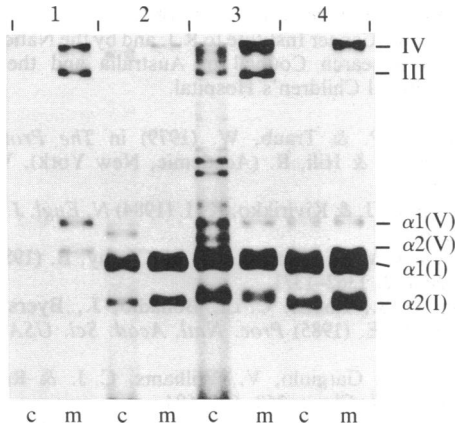

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of [^{3}H]-proline-labeled pepsin-resistant collagen produced by control and transfected cell lines. The cell layer (c) and medium (m) samples were analyzed separately and loaded quantitatively into each well. Samples were unreduced. Lanes 1, homozygous Mov-13 cell line M13a; lanes 2, wild-type cell line; lanes 3, Mov-13 cell line M13-SV-H22 transfected with the human proα1(I) gene under SV40 control; lanes 4, Mov-13 cell line M13-M5 transfected with the mouse proα1(I) gene. The electrophoretic migration positions of type I collagen α1(I) and α2(I) chains, type V collagen α1(V) and α2(V) chains, and type III and IV collagens are indicated.

However, the α1(I) and α2(I) chains in the human–mouse hybrid molecules migrated more slowly on electrophoresis than the control mouse α chains. Slow migration was not apparent when unhydroxylated procollagen was analyzed by gel electrophoresis (Fig. 4), suggesting that the abnormal electrophoretic migration of the α1(I) and α2(I) chains in the human–mouse hybrid molecules may be due to altered posttranslational hydroxylation of lysine. Collagen proline hydroxylation was similar in all transfected cell lines analyzed (data not shown).

The amounts of α1(I) and α2(I) chains were quantitated from a combination of pepsin-resistant collagen in the medium and cell layer fractions to measure the levels of α chains assembled into helical collagen molecules in transfected cells as a proportion of control levels (Table 1). The results showed that Mov-13 cells transfected with the human and mouse genes synthesized high levels of both α1(I) and α2(I) chains, which were close to the levels made by control cells. Since the mRNA levels were also approximately equal to those in the wild-type embryo fibroblasts (Fig. 2), the data indicated

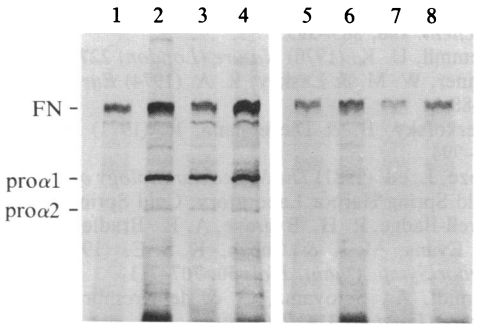

FIG. 4. NaDodSO$_4$/polyacrylamide gel electrophoresis of unhydroxylated procollagen from the cell layers labeled with [^{3}H]proline in the presence of α,α′-dipyridyl. Samples were loaded prior to (lanes 1–4) and after digestion with bacterial collagenase (lanes 5–8). All samples were reduced with 5% (vol/vol) 2-mercaptoethanol. Lanes 1 and 5, M13a cell line; lanes 2 and 6, wild-type cell line; lanes 3 and 7, M13-SV-H22 cell line; lanes 4 and 8, M13-M5 cell line. The migration positions of the collagenase-sensitive proα1(I) and proα2(I) chains of type I collagen and of fibronectin (FN) are indicated.

Table 1. Pepsin-resistant type I collagen levels in transfected cell lines

Cell line	α1(I) chain, % control	α2(I) chain, % control	α1(I)/α2(I)
Wild type	100	100	3.0
M13	0	0	0
M13-SV-H22	69	125	1.6
M13-M5	77	100	2.3

The pepsin-resistant α1(I) and α2(I) chain protein levels were quantitated by excision of the bands from electrophoretic gels (Fig. 3), hydrolysis, and scintillation spectroscopy. Values represent the means of at least four replicate determinations and are expressed as a percentage of control values.

that the amount of functional hybrid molecules was directly related to the transcriptional activity of the inserted gene.

The inserted human and mouse α1(I) chains associated into heterotrimers {[α1(I)]$_2$ α2(I)} with the endogenous α2(I) chains, as shown by the approximate 2:1 ratio between α1(I) and α2(I) chains (Table 1). These data demonstrated that sufficient compatibility existed between the human and mouse α chains to enable correct association and helix formation. The inserted proα1(I) gene products did not preferentially form homotrimers [α1(I)]$_3$, as would have been expected in the absence of sufficient compatibility. In the control cells, a ratio of 3.0 between α1(I) and α2(I) chains suggested that some α1(I) trimers were produced by these cells.

DISCUSSION

In this study we have investigated whether human proα1(I) chains can associate with a mouse proα2(I) chain to form functional type I collagen. We have introduced a cosmid clone of the human proα1(I) gene into homozygous Mov-13 cells that carry functional deletions of both endogenous proα1(I) genes. The human gene under the transcriptional control of its own promoter and 1.5-kb upstream sequences was poorly expressed in the transfected mouse cells. Likewise, only low expression of a human α1(II) collagen was observed in transgenic mice (29). In contrast, different cosmid clones coding for the mouse proα1(I) collagen gene containing either 4 kb, 2.5 kb, or 1 kb of the 5′ flanking mouse sequences were transcribed at high levels in Mov-13 cells. Results obtained with the mouse proα2(I) collagen gene either transfected into cells in tissue culture (30, 31) or introduced into transgenic mice (32) have similarly demonstrated that only 1–2 kb of flanking sequences are sufficient for tissue-specific expression of this gene. It therefore appears that the human regulatory sequences of the proα1(I) collagen gene function poorly in mouse cells. In contrast, we observed high expression comparable to the level of the endogenous mouse gene when the transfected human gene was under the transcriptional control of the SV40 promoter. RNA transfer blot analysis revealed that full-length proα1(I) mRNA was transcribed from the transfected gene. Some transfected cell clones synthesized both proα1(I) mRNAs, whereas others expressed only the lower molecular weight RNA species. It is possible that the transfected gene was truncated in the latter cell clones, thus eliminating the normal poly(A) addition site at the 3′ end of the larger mRNA. Since these cells produced apparently normal procollagen chains independent of whether both mRNA species were present or not, the data indicate that the distribution of the proα1(I) mRNA into two forms may be of little functional consequence (15).

Transfection of Mov-13 cells with either a mouse or human proα1(I) gene resulted in the production of proα1(I) procollagen chains that were able to associate with procollagen chains expressed by the endogenous proα2(I) gene and form

collagen molecules that were secreted into the medium. Functional expression of the inserted proα1(I) gene rescued the proα2(I) gene products from rapid degradation and allows secretion of normal levels of collagen proα2(I) chains. Appearance of pepsin-stable triple-helical heterotrimers of α1(I) and α2(I) chains in the normal $[\alpha_1(I)]_2$ α2(I) configuration is good evidence for the normal structure of the gene product from the inserted proα1(I) genes. Mov-13 cells transfected with the mouse proα(I) gene synthesized and secreted type I collagen molecules that are similar to the type I collagen produced by control embryo fibroblasts by several criteria, including α1(I) and α2(I) chain association, degree of secretion, stability of the triple helix to pepsin treatment, and migration of pepsin-treated collagen α chains on gel electrophoresis.

Cells transfected with the human proα1(I) gene also synthesized and secreted stable heterotrimers of α1(I) and α2(I) chains, but in this case abnormally slow electrophoretic migration of the human α1(I) chain and the endogenous mouse α2(I) chain was found. Inhibition of posttranslational hydroxylation of lysine and proline resulted in comigration of the human and mouse α chains, suggesting that posttranslational overmodification caused the altered electrophoretic migration. In many cases of lethal perinatal OI slow electrophoretic migration of collagen α chains is also observed due to lysine overhydroxylation and an increased glycosylation of hydroxylysine residues (8, 9). It has been proposed that in patients with lethal perinatal OI, lysine residues in the collagen α chains are exposed to the action of modifying enzymes for longer periods of time as a result of mutations that delay the association of α chains into triple-helical structures (8). Though there is considerable overall homology between mouse and human α1(I) chains (11, 16), a number of amino acid sequence differences between the mouse and human chains (21, 33) may be sufficient to reduce the rate of chain association and cause an abnormality in the degree of posttranslational modification.

Our data show that stable triple-helical type I collagen molecules are secreted into the extracellular environment by transfected Mov-13 cells. It is not clear, however, whether these molecules can form normal collagen fibrils and whether these interact with other components to form normal extracellular matrices *in vivo*. Though the type I collagen produced by the cell lines containing the mouse gene is likely to be normal, human–mouse hybrid type I collagen has properties similar to the abnormal collagen produced by patients with lethal perinatal OI. The introduction of the human gene into Mov-13 embryos will directly test whether the human–mouse interspecies heterotrimer can function normally in the animal. Preliminary evidence does indeed indicate that the heterotrimer can function *in vivo* because insertion of the human proα1(I) gene into the germ line of Mov-13 mice partially rescues the lethal phenotype of homozygous embryos (unpublished).

This study provides evidence for the functional expression of a transfected gene that codes for one subunit of a multisubunit protein and demonstrates that human proα1 and mouse proα2 chains can associate to form stable type I collagen. Similarly, it has been shown that apparently functional microtubules can be generated from association of interspecies α-tubulin subunits (34). The ability to insert the proα1(I) gene into Mov-13 cell lines and to obtain functional expression should now make it feasible to alter the proα1(I) gene by site-directed mutagenesis and to study the effect of these specific mutations on collagen synthesis, assembly, and function.

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