Rescue of Type I Collagen-Deficient Phenotype by Retroviral-Vector-Mediated Transfer of Human pro α 1(I) Collagen Gene into Mov-13 Cells

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A full-length cDNA clone corresponding to the human pro α 1(I) collagen gene was isolated and inserted into a retrovirus vector. Cell lines were obtained which produced recombinant viruses transducing the collagen cDNA (HUC virus). To test whether the transduced cDNA was functional, Mov-13 mouse cells were infected with the virus. These cells do not produce any type I collagen due to an insertional mutation of the pro α 1(I) gene which blocks transcription. While normal amounts of pro α 2(I) RNA were synthesized, no α 2(I) collagen chains were detectable in the mutant Mov-13 cells. Infection with HUC virus, however, resulted in the production of stable type I collagen, which was secreted into the medium. Analysis of pepsin-resistant proteins indicated that interspecies heterotrimers consisting of human α 1(I) and mouse α 2(I) collagen chains were secreted by the infected Mov-13 cells. Our results show that pro α (I) collagen chains from species as distant as human and mouse can associate to form stable type I collagen. The availability of a retrovirus vector transducing a functional pro α 1(I) collagen gene combined with the Mov-13 mutant system should enable us to study the effect of specific mutations on the synthesis, assembly, and function of type I collagen, not only in tissue culture but also in the animal.

Type I collagen, a major protein of the extracellular matrix, is composed of one α 2(I) and two α 1(I) chains which are associated in a triple helix. The α chains are approximately 1,000 amino acids in length and consist largely of repeated triplets of the amino acids Gly-X-Y, in which X and Y are frequently proline or hydroxyproline. The α chains are synthesized as procollagen precursor molecules, which align at their carboxy termini and form a triple helix which propagates towards the amino termini. Triple-helical molecules are secreted by the cell, and the extension peptides are removed prior to collagen fibril formation (for a review, see reference 23).

The analysis of certain types of the human inherited disorder osteogenesis imperfecta (OI) has begun to yield genetic information on the mechanism of helix formation and on functional domains of the pro α (I) collagen molecules. Functional deletion of one pro α 1(I) gene reduces the total amount of type I collagen by 50% and results in a disorder designated OI type I (2), which is characterized by brittle bones, thin skin, and blue sclerae. A perinatal lethal form of the disease, designated OI type II, has been shown in several cases to be due to structural alterations in the helical region of the α 1(I) gene (5). Some mutations of this type have been studied in detail and found to be small deletions (3, 7) or point mutations (9, 28). It has been proposed that alterations in the α chains can affect the correct formation of the triple helix and thereby disturb the maturation and secretion of functional type I collagen. These patients demonstrate that even very subtle alterations in collagen structure can result in a drastic loss of biological function (5).

The Mov-13 substrain of mice (15) is a model system for the study of type I collagen structure and function. In this mouse strain, transcription of the pro α 1(I) collagen gene is blocked by the integration of a Moloney murine leukemia

virus (M-MuLV) into the first intron (14). Mov-13 heterozygotes contain 50% of the normal amount of type I collagen and are therefore analogous to OI type I patients (24). In contrast to OI type I patients, heterozygous Mov-13 mice are phenotypically indistinguishable from wild-type littermates, indicating that mice and humans require different levels of collagen expression for normal function. Animals homozygous for the Mov-13 mutation lack type I collagen completely and die at midgestation (25). Cell lines derived from homozygous embryos transcribe normal amounts of pro α 2(I) collagen RNA (25), but contain no functional pro α 2(I) chains (21). This is most likely due to rapid degradation of the pro α 2(I) chains, which cannot form stable triple helices in the absence of pro α 1(I) chains (30). We used homozygous Mov-13 cell lines as a test system for deriving a functional pro α 1(I) collagen cDNA. Here we describe the cloning of the complete human pro α 1(I) collagen cDNA and the construction of a retrovirus vector capable of transducing the gene. The pro α 1(I) collagen cDNA is functional because infection of Mov-13 cells with the vector rescued the mouse pro α 2(I) chain and restored type I collagen expression. Our experiments demonstrate that the human pro α 1(I) chain can associate with the mouse pro α 2(I) chain to form a stable interspecies collagen I heterotrimer, which is secreted into the extracellular space.

MATERIALS AND METHODS

Cell culture, transfection, viral infection, and selection. The derivation of Mov-13 homozygous embryo fibroblast cell lines has been described elsewhere (24). M13 is an uncloned fibroblast line derived from a day-12 Mov-13 homozygous embryo, which was immortalized by simian virus 40 (SV40) transformation. All tissue culture cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% calf serum. Cells were transfected by the method of Graham and Van der Eb (13) or infected by virus supernatant

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FIG. 1. Diagram of the HUC virus (pHUCV). The vector is pLJ(DOL-), with the replacement of the 3' LTR of MPSV from the *Clal* site into the 3'-flanking region. All *Clal*, *BamHI*, *XbaI*, and *HindIII* sites are shown. The *BamHI* and *Sall* cloning sites of the vector have been destroyed by insertion of the human pro α 1(I) collagen cDNA. Arrows indicate the expected transcripts originating from the 5' LTR and the SV40 promoters.

containing 4 μ g of Polybrene per ml. When selecting for the presence of the human collagen virus, ψ -2 (22) and ψ -AM (11) cells were selected in 1 mg of G418 (Gibco Laboratories) per ml, and M13 cells were selected in 0.3 mg of G418 per ml.

α 1(I) collagen cDNA isolation and construction of a retroviral vector. A human fibroblast (SV80) cDNA library in bacteriophage λ gt10 (provided by D. Wolf) was screened for large α 1(I) collagen cDNA inserts by hybridizing replica plates with cloned fragments from the extreme 5' and 3' ends of the mouse gene (14, 24). The largest clone isolated lacked an 80-base-pair (bp) region at the 3' end of the coding region. To provide a complete coding region, a fragment from the partial-length human cDNA clone Hf677 (8) was fused at the 3' end.

The retroviral vector used for transduction of the cDNA was pLJM. This is a modification of the vector pLJ(DOL-) (17). pLJ(DOL-) is designed to transcribe DNA regions cloned into unique *BamHI* and *Sall* cloning sites from the 5' long terminal repeat (LTR) promoter. M-MuLV splicing signals have been removed to avoid the possibility of aberrant spliced transcripts. pLJM was made by replacing the 3' LTR of pLJ(DOL-) with that of the myeloproliferative sarcoma virus (MPSV). This was achieved by exchanging a fragment between the *Clal* site and the *HindIII* site in the polyomavirus early region of pLJ(DOL-) (17) for a fragment of the MPSV proviral clone p18-663 between the *Clal* site and an *HindIII* site in the 3'-flanking region (16, 27). As viral replication duplicates the U3 region of the 3' LTR at the 5' end, transcription of cloned inserts is directed by the MPSV proviral LTR promoter.

The human α 1(I) collagen cDNA was ligated to *BclI* and *XhoI* linkers at the 5' and 3' ends, respectively, and cloned into pLJM between the *BamHI* and *Sall* sites (Fig. 1).

Nucleic acid blot analysis. Total cellular RNA was prepared by the method of Auffray and Rougeon (1). RNA samples which had been denatured with glyoxal and restriction digests of genomic DNA were separated on agarose gels, transferred to GeneScreen Plus nylon membrane (New England Nuclear Corp.), and hybridized to 32 P-labeled probes by standard methods.

Preparation and analysis of in vivo-labeled collagen. Protein analyses were performed essentially as described by Bateman et al. (4). Cells were plated onto 6-cm-diameter dishes and grown until confluent. The medium was then replaced with 5 ml DMEM, 10% dialyzed fetal calf serum, 2 mM glutamine, and 150 μ M ascorbic acid. After 2 h, 50 μ Ci of

[3 H]proline (Amersham Corp.) and β -aminopropionitrile to a final concentration of 0.1 mM were added, and incubation was continued for 16 h. The medium was collected, and the cell layer was rinsed with 2 ml of isotonic HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). The rinse and medium were combined and chilled on ice, and protease inhibitors were added, with phenylmethylsulfonyl fluoride to 0.1 mM and EDTA to 25 mM. Collagen was precipitated in 25% saturated $(\text{NH}_4)_2\text{SO}_4$ for 16 h at 4°C, pelleted, dissolved in 50 mM Tris hydrochloride (pH 7.5)–150 mM NaCl, precipitated in 75% ethanol, pelleted, washed in 80% ethanol, lyophilized, dissolved in 0.5 M acetic acid containing pepsin (Boehringer Mannheim) (100 μ g/ml), and incubated at 4°C for 6 h. Samples were lyophilized, dissolved in gel buffer, and analyzed by 5% polyacrylamide-sodium dodecyl sulfate (SDS) gel electrophoresis (19), followed by autoradiography.

RESULTS

Construction of an α 1(I) collagen-transducing retrovirus. pHUC (human collagen) is a cDNA clone corresponding to the complete 4.8-kilobase (kb) human pro α 1(I) collagen mRNA, which was isolated from a cDNA library of human SV80 fibroblasts. Restriction mapping and detailed comparison with data for human genomic and partial-length cDNA clones (6, 8) showed that pHUC contained all coding sequences of the pro α 1(I) collagen gene. The 5' end of pHUC was found to include sequences up to approximately 30 bp upstream of the start of translation. The 3' end, which was derived from the human pro α 1(I) collagen cDNA clone Hf677 (8), is 20 bp upstream of the polyadenylation signal for the 4.8-kb mRNA (6).

To test whether the cDNA was functional, it was cloned into a retrovirus vector (pLJM) capable of expressing inserted gene sequences in host mouse cells. This construct (pHUCV) was designed so that the collagen cDNA is transcribed from the 5' proviral MPSV LTR promoter (27) and the neomycin phosphotransferase gene, which confers resistance to the drug G418 (10), from the SV40 promoter. Both mRNAs terminate in the 3' LTR. The MPSV LTR promoter was chosen because of evidence that it is more active than the M-MuLV LTR promoter in certain cell types, notably embryonal carcinoma cells (12, 26). The structure of pHUCV and the predicted transcripts are given in Fig. 1.

Derivation of HUC virus-producing cell lines. To obtain a virus producer line, pHUCV DNA was transfected into the packaging cell line ψ -2 (22) and G418-resistant clones were isolated. Thirty-seven transfected ψ -2 clones were screened for virus production by infection of NIH 3T3 cells with supernatant and G418 selection. One cell line (ψ -2HUC2) producing 5×10^4 G418^r CFU/ml was selected for further study.

Mov-13 cells produce infectious M-MuLV and are therefore resistant to superinfection by an ecotropic recombinant retrovirus. This block to infection can be overcome by pseudotyping the vector with an amphotropic helper virus. We therefore infected ψ -AM2275 cells, which carry an amphotropic helper virus lacking the packaging signal (11), with ψ -2HUC2 supernatant, isolated G418-resistant clones, and tested for virus production on NIH 3T3 and human SV80 cells. The supernatants of individual ψ -AMHUC clones gave equal titers on the human and mouse cells and varied between 5×10^2 and 5×10^3 CFU/ml.

To test whether full-length viral RNA was synthesized and passed as infectious virus, we infected the Mov-13 cell line

FIG. 2. Passage of the HUC virus by ψ -AM producer clones. Total RNA from five ψ -AMHUC producer clones and uncloned populations of M13 cells infected by each clone was hybridized to pHUC collagen cDNA. Lanes: A, ψ -2HUC-2; B, C, D, E, F, ψ -AMHUC-1, -2, -3, -4, and -5, respectively; lanes G, H, I, J, K, M13 populations infected with ψ -AMHUC-1, -2, -3, -4, and -5, respectively.

M13 with supernatants from ψ -AMHUC clones and selected for G418 resistance. RNA was isolated from different ψ -AMHUC producer clones and from infected M13 cell populations. Northern blot (RNA blot) analysis revealed that some ψ -AMHUC cell lines expressed full-length viral RNA, while others expressed shorter viral RNA (Fig. 2, lanes B to F). Each infected M13 population expressed viral RNA which was the same length as that of the ψ -AMHUC clone used for infection. These results indicate that the original transfected ψ -2HUC-2 cell line must have produced full-length and deleted viruses and that each virus was stably passed from cell to cell.

The proviral copy number in individual M13 clones infected by the full-length HUC virus was determined by Southern analysis of *Hind*III-digested genomic DNA. This enzyme cleaves once within the HUC virus. Therefore, each proviral integration will yield a single fragment corresponding to the 5' part of the provirus and flanking host sequence when hybridized to labeled pHUC DNA (Fig. 1). Four fragments corresponding to the endogenous $\alpha 1(I)$ collagen gene were detected in uninfected M13 cells (Fig. 3A, lane A). A single additional band was seen in infected cells (lanes B and C), indicating the presence of a single HUC provirus. Similar results were obtained after *Eco*RI digestion (data not shown). Single-copy integrations were found in every clone analyzed.

The integrity of the provirus in M13HUC-1 and -5 cells was investigated by comparing internal proviral restriction fragments covering the entire cDNA and the 5' LTR promoter with those of cloned pHUCV DNA. Figure 3B shows an *Xba*I-*Bam*HI digest of genomic DNA from uninfected M13 cells, M13HUC-1 and -5 cells, and pHUCV DNA probed with pHUC. Two proviral fragments with the predicted sizes of 3.5 and 2 kb were seen in the infected cell lines and in the pHUCV plasmid DNA, indicating that the virus had undergone no major structural alteration. This was confirmed with *Xba*I-*Eco*RI and *Sst*I-*Eco*RI restriction digests (data not shown).

$\alpha 1(I)$ collagen RNA in infected Mov-13 cells. Pro $\alpha 1(I)$ collagen RNA synthesized by HUC virus-infected Mov-13 cells was quantitated by Northern blot analysis. Figure 4

shows the analysis of total RNA from seven infected Mov-13 clones. M13HUC-1, -2, -3, -5, and -6 cells were infected with a full-length virus and M13HUC-4 and -7 with a partially deleted virus. The cell clones produced RNA of either 8.8 or 8.5 kb, which hybridized to the collagen and *neo* probes, corresponding to the genomic viral RNA. A 3.4-kb transcript which hybridized only to the *neo* probe corresponded to RNA initiated at the internal SV40 promoter (Fig. 1). No spliced RNA was detected, as the retroviral vector used lacked the splicing signals (17).

The amount of the 8.8-kb viral RNA in the M13HUC clones was compared by densitometry with the 4.8- and 5.8-kb collagen RNAs produced by NIH 3T3 cells. The amount of RNA in each lane was standardized by hybridization to a rat α -tubulin probe (20). This analysis showed that in different clones the concentration of viral RNA varied between 40 and 150% of the total pro $\alpha 1(I)$ collagen RNA in NIH 3T3 cells. These results show that the transduced human pro $\alpha 1(I)$ collagen under the control of MPSV LTR promoter was transcribed as efficiently as the endogenous gene. Two clones were selected for further analysis: M13HUC-1, which produced approximately the same quantity of viral RNA as the total pro $\alpha 1(I)$ collagen RNA from NIH 3T3 cells, and M13HUC-5, which produced approximately 40% of that amount.

$\alpha 1(I)$ collagen in infected Mov-13 cells. We analyzed whether the human pro $\alpha 1(I)$ collagen RNA expressed in infected Mov-13 cells was translated into pro $\alpha 1(I)$ collagen chains, which could associate with the endogenous pro $\alpha 2(I)$ chains. Although Mov-13 homozygotes are known to transcribe the pro $\alpha 2(I)$ collagen gene normally (25), no $\alpha 2(I)$

FIG. 3. HUC provirus in infected Mov-13 clones. (A) Proviral copy number in two Mov-13 clones. *Hind*III-cut genomic DNA was hybridized to pHUC. Lanes: A, uninfected M13 DNA; B and C, M13HUC-1 and -5, respectively. Endogenous $\alpha 1(I)$ collagen *Hind*III fragments are indicated. (B) Proviral integrity in two Mov-13 clones. *Xba*I-*Bam*HI-cut genomic DNA was hybridized with pHUC. Lanes: A, uninfected M13; B and C, M13HUC-1 and -5, respectively; D, pHUCV plasmid DNA. Arrows indicate the endogenous $\alpha 1(I)$ collagen and HUC proviral fragments.

FIG. 4. HUC virus transcripts in infected Mov-13 clones. Total RNA from Mov-13 clones infected by ψ -AMHUC producers was hybridized to pHUC, a *neo* probe, and a rat α -tubulin probe as an internal standard for RNA quantitation. Lanes: A, B, C, D, E, F, G, M13HUC-1, -2, -3, -4, -5, -6, and -7, respectively; H, ψ -2HUC-2; I, NIH 3T3. The upper autoradiograph shows hybridization to pHUC. The middle autoradiograph is the same filter rehybridized to a *neo* probe. The bottom strip shows the intensity of the α -tubulin signal in each lane.

chains are detectable by immunostaining (21). In the absence of pro α 1(I) collagen expression, pro α 2(I) collagen chains are unable to form stable triple helices and are rapidly degraded (30). The presence of pepsin-resistant, secreted collagen is therefore a good indication of functional association of the pro α chains and the formation of stable triple helices.

Figure 5 shows a polyacrylamide gel analysis of [3 H]proline-labeled pepsin-resistant protein secreted into the medium by M13HUC-1 and -5 cells, by uninfected M13 cells, by M13 cells transfected with the mouse pro α 1(I) collagen gene, and by NIH 3T3 cells. Several pepsin-resistant bands were resolved. Previous work identified the different bands as the α 1 and α 2 chains of type I and type V collagens (18, 24). Under the gel conditions used, the α 2(V) collagen band migrated only slightly above the α 1(I) chain; however, the presence or absence of the α 2(I) chain provided clear and unambiguous evidence for the presence or absence of type I collagen (18, 24).

Lane A shows M13-M5, which is an Mov-13 homozygous cell line transfected with a mouse pro α 1(I) collagen construct; lane B shows the parental untransfected line M13. Comparison of these two lanes clearly shows the absence of α 1 and α 2 chains in the Mov-13 cell line and the presence of both in the transfected cell line. The cell line M13-M5 has been analyzed by Schnieke et al. (24) and shown to produce normal type I collagen. These lanes served as controls for the virus-infected Mov-13 cells. Lanes C and D (M13HUC-1 and -5, respectively) show the presence of α 1 and α 2(I)

collagen chains, which were absent from the parental uninfected M13 cell line (lanes B and E), but present in the NIH 3T3 control (lane F). Our results demonstrate that helix formation occurred between the endogenous mouse pro α 2(I) chains and the human pro α 1(I) chains produced by the retroviral-vector-transduced gene. Quantitation of the proteins showed that the amount of type I collagen secreted by the infected Mov-13 cells and NIH 3T3 control cells correlated with the level of pro α 1(I) collagen RNA. These results show that RNA transcribed by the transduced gene was as efficiently translated as the endogenous pro α 1(I) collagen RNA.

Additional evidence for the production of stable type I collagen was obtained by immunocytochemical staining of infected Mov-13 cells, uninfected Mov-13 cells, and NIH 3T3 cells with anti-type I collagen antiserum by a method similar to that used by Löhler et al. (21). As shown previously, the Mov-13 cells did not stain for type I collagen (21). However, both virally infected Mov-13 cells and NIH 3T3 cells showed a strong staining reaction (data not shown).

DISCUSSION

In this study, we demonstrate that type I collagen synthesis can be restored in homozygous Mov-13 embryo fibroblasts by retroviral transduction of the human pro α 1(I) collagen cDNA. Full-length human cDNA was inserted into the retroviral vector pLJM under the control of the MPSV LTR promoter. Infected Mov-13 cells contained intact single copies of the HUC provirus and transcribed human pro α 1(I) collagen viral RNA at levels comparable to that of the endogenous mouse pro α 1(I) collagen gene in NIH 3T3 cells. The viral RNA was translated into pro α 1(I) collagen chains, which combined with endogenous pro α 2(I) chains to form stable type I collagen, which was secreted into the medium. Pro α 1(I) collagen cDNA is the largest gene that has been introduced into cells by retroviral infection to date. The fact that such a large and internally repetitive gene sequence can be stably passed as a retrovirus is encouraging for the general use of viral vectors in gene transfer.

FIG. 5. Collagen produced by infected Mov-13 clones. Pepsin-resistant, [3 H]proline-labeled collagen secreted into the medium by five cell lines separated on a 5% SDS-polyacrylamide gel under reducing conditions, visualized by autoradiography. Lanes: A, Mov-13 homozygous cell line M13 transfected with a mouse pro α 1(I) collagen genomic clone; B, untransfected M13; C and D, M13HUC-1 and -5, respectively; E, uninfected M13; F, NIH 3T3. The positions of the α chains of type I and V collagens are marked by arrows.

The strongest evidence that the HUC virus transduced a functional pro α 1(I) collagen RNA derived from infection of Mov-13 cells, which are unable to synthesize endogenous pro α 1(I) collagen RNA. While these cells produce normal amounts of pro α 2(I) RNA (25), no stable pro α 2 chains are present, because they are rapidly degraded when not associated with pro α 1 chains in a triple helical structure (30). Infection of Mov-13 cells with HUC virus resulted in the formation and secretion of stable type I collagen consisting of human α 1 and mouse α 2 chains. Production of stable type I collagen was confirmed by specific immunocytochemical staining of infected Mov-13 cells with a type I collagen antibody. These data therefore indicate that pro α collagen chains from species as distant as mouse and human can associate and form stable type I collagen molecules. This confirms recent results obtained after transfection of a genomic clone of the human pro α 1(I) collagen gene into Mov-13 cells (24). Similar to the previous results, the endogenous mouse pro α 2 chains were rescued by the human pro α 1 chain.

The parameters which are important for proper triple helix formation are poorly defined. Up to now, the only genetic approach to an understanding of the interactions between the pro α chains which are crucial for functional triple-helix formation has been the analysis of patients with lethal OI. Recently it has been shown that in two cases, point mutations in the helical region of the α 1 chain resulted in the perinatal lethal form of the disorder (9, 28; J. Bateman, personal communication). Analysis of these two patients suggested that a single substitution of glycine in a Gly-X-Y-triplet interferes with the folding of the triple helix, which is essential for its proper function. However, it would be impractical to perform genetic analysis of the regions of the α chains which are important for correct assembly on the basis of rare, sporadic mutations in humans. A more efficient and systematic approach to structure-function analysis would be site-directed mutagenesis of cloned genes and subsequent introduction into appropriate human cells. This is difficult with the existing genomic clones of pro α 1(I) collagen (6) because of the size of the gene and the poor transfectability of human cell strains. The HUC retrovirus we describe is suitable for such an analysis, as mutations can be made in the relatively small cDNA and mutant collagen genes can be introduced efficiently into cell types of interest by viral infection.

The present experiments have established that transduction of the human pro α 1(I) collagen gene results in synthesis and secretion of stable type I collagen and thus rescues the mutant phenotype of Mov-13 cells in tissue culture. This work has not established whether the mouse-human hybrid collagen can participate in the formation of a functional extracellular matrix in the animal. The experimental introduction of a human pro α 1(I) collagen genomic clone into the germ line of Mov-13 mice has, however, resulted in the rescue of the lethal phenotype, at least until birth (unpublished data), demonstrating that the hybrid molecule can function *in vivo*. The availability of a retroviral vector capable of transducing the pro α 1(I) collagen gene opens the possibility of rescuing Mov-13 homozygous embryos by restoring the gene function to somatic cells. For this embryos will be infected *in utero* with the viral vector (29) prior to the time of death. Survival of homozygous embryos beyond day 14 of gestation would indicate rescue of the lethal phenotype. This method of "gene therapy" may be of potential medical relevance, as it would involve gene transfer into somatic cells only and no manipulation of the germ line.

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