

# Reduction of Type V Collagen Using a Dominant-negative Strategy Alters the Regulation of Fibrillogenesis and Results in the Loss of Corneal-Specific Fibril Morphology

Jeffrey K. Marchant, Rita A. Hahn, Thomas F. Linsenmayer, and David E. Birk

Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts 02111

**Abstract.** A number of factors have been implicated in the regulation of tissue-specific collagen fibril diameter. Previous data suggest that assembly of heterotypic fibrils composed of two different fibrillar collagens represents a general mechanism regulating fibril diameter. Specifically, we hypothesize that type V collagen is required for the assembly of the small diameter fibrils observed in the cornea. To test this, we used a dominant-negative retroviral strategy to decrease the levels of type V collagen secreted by chicken corneal fibroblasts. The chicken  $\alpha 1(V)$  collagen gene was cloned, and retroviral vectors that expressed a polycistronic mRNA encoding a truncated  $\alpha 1(V)$  minigene and the reporter gene *LacZ* were constructed. The efficiency of viral infection was 30–40%, as determined by assaying  $\beta$ -galactosidase activity. To assess the expression from the recombinant provirus, Northern analysis was performed and indicated that infected fibroblasts expressed high steady-state levels of retroviral mRNA. Infected cells

synthesized the truncated  $\alpha 1(V)$  protein, and this was detectable only intracellularly, in a distribution that colocalized with lysosomes. To assess endogenous  $\alpha 1(V)$  protein levels, infected cell cultures were assayed, and these consistently demonstrated reductions relative to control virus-infected or uninfected cultures. Analyses of corneal fibril morphology demonstrated that the reduction in type V collagen resulted in the assembly of large-diameter fibrils with a broad size distribution, characteristics similar to fibrils produced in connective tissues with low type V concentrations. Immunoelectron microscopy demonstrated the amino-terminal domain of type V collagen was associated with the small-diameter fibrils, but not the large fibrils. These data indicate that type V collagen levels regulate corneal fibril diameter and that the reduction of type V collagen is sufficient to alter fibril assembly so that abnormally large-diameter fibrils are deposited into the matrix.

THE characteristics of different extracellular matrices derive in large part from the synthesis, assembly, and deposition of collagen molecules and their organization into unique macromolecular structures. These collagen assemblies confer specialized properties to the extracellular matrix in which they are found. The cornea, for example, has small, uniform diameter fibrils organized into orthogonal lamellae; these characteristics permit this tissue to be strong yet transparent.

The synthesis of collagen molecules and their association to form fibrils requires a number of sequential post-translational events that are common to all fibrillar collagens (19). These include intracellular processes such as hydroxylation and glycosylation, and extracellular ones such as procollagen processing and cross-linking (14, 19, 30). Fibril assembly may be modified through the association of different fibrillar collagens to form heterotypic fibrils (3, 5, 15, 18, 24), the addition of proteoglycans to

form collagen-proteoglycan heteropolymers (33, 39), or by the addition of fibril-associated collagens to fibrils (19). Alternative splicing of collagens also can occur in a tissue-specific manner and confer different properties to fibrils (27, 32, 35, 37). Tissue-specific variations in all these events contribute to the diversity of collagen structures observed in different extracellular matrices.

Fibril assembly is a multistep process and, as such, there are numerous points where fibril characteristics, i.e., diameter, may be regulated. Propeptide processing represents one such checkpoint. The C-propeptides of procollagen molecules are cleaved in the extracellular matrix before incorporation of collagen monomers into fibrils, and therefore C-propeptidase activity provides an early rate-limiting step in fibril formation (14, 30). N-propeptidase activity varies with collagen substrates, being rapid with type I collagen yet limited with type III (28, 31). A unique example of this propeptidase regulation exists with heterotypic type I/V fibrils. Type I collagen in these fibrils is fully processed while type V is only partially processed, using a distal cleavage site that leaves the bulk of the noncollagenous amino-terminal domain intact (20).

Please address all correspondence to David E. Birk, Department of Anatomy and Cellular Biology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Tel.: (617) 636-0301; Fax: (617) 636-8577.

Type V collagen is copolymerized into heterotypic type I/V fibrils in a molecular arrangement that leaves the amino-terminal domain exposed on the surface and the major triple-helical portion of the molecule located within the fibril (20). Between the major triple helix and this amino-terminal domain lies a "hinge region" and three small triple-helical domains that we have previously suggested permit the extension of the amino-propeptide through the hole zone and onto the fibril surface (20). This structural arrangement observed in heterotypic type I/V fibrils exists with other fibrils as well. Type II collagen fibrils, for example, contain type XI collagen molecules, and these are positioned in a way that the amino-terminal domains project onto the fibril surface. As with type V collagen, the triple-helical domains are within the fibril (24, 29, 42). The similarities between collagen types V and XI extend beyond a structural resemblance: in certain tissues (e.g., vitreous, bone) type V collagen molecules can be comprised of both type V and type XI  $\alpha$  chains, suggesting that type XI chains are able to substitute for type V chains (12). Given these structural and functional similarities, it has been suggested that these two collagens comprise part of a larger fibrillar collagen family, one that has a predominantly regulatory rather than structural function (12, 22).

Fibril diameter is related to the concentration of type V collagen within heterotypic type I/V fibrils, and it is the amino-terminal domain that appears to confer this regulatory property. Previous self-assembly studies have demonstrated that in vitro, the levels of type V collagen incorporated into type I/V fibrils is inversely proportional to fibril diameter: the higher the concentration of type V, the smaller the diameter. It also was shown that if the amino-terminal domain is removed, diameter regulation is abolished. It is hypothesized that the presence of the amino-terminal surface component is able to block accretion of collagen monomers and, hence, limit growth (20). Although these in vitro studies demonstrated a strong relationship between type V concentration and fibril diameter, the smallest diameters achieved were larger than those observed in situ (4).

To test in vivo the hypothesis that the concentration of type V collagen is sufficient to control diameter, we used a dominant-negative retroviral approach to alter the levels of  $\alpha 1(V)$  collagen and thus perturb the type I/V collagen ratios within heterotypic fibrils. We reasoned that the synthesis of a truncated  $\alpha 1(V)$  chain, possessing only the C-propeptide and a portion of the triple helix, would be functional in chain assembly and yield hybrid triple-helical molecules composed of both truncated and endogenous  $\alpha$  chains. These would be targeted to degradative pathways and thereby reduce the concentration of type V collagen (30, 41). The results demonstrate that reduced type V collagen levels in corneal fibroblasts lead to the assembly and deposition of abnormally large fibrils into the extracellular matrix.

## Materials and Methods

### Cloning of $\alpha 1(V)$ Collagen Carboxyl Terminus

The carboxyl terminus of  $\alpha 1(V)$  collagen was cloned using 3' RACE procedures using 14-d cornea total RNA as the template. A poly(T)-adapter

primer was used in the initial reverse-transcription reaction to generate cDNA. This was followed by PCR amplification using the same 3' primer and a 5' primer specific to a region within the triple-helical domain of the  $\alpha 1(V)$  mRNA (5' GGGAAGACAGGCCCAATCGGCC 3'). PCR-amplified cDNA was cloned into the TA vector (Invitrogen, San Diego, CA), and purified DNA was subjected to sequence analysis using the dsDNA Cycle Sequencing System (GIBCO BRL, Gaithersburg, MD).

### Replication-defective Retroviral Vectors

Retroviral vectors were kindly provided by Dr. Takashi Mikawa (Cornell University Medical College, New York, NY). These vectors are derived from spleen necrosis virus (SNV)<sup>1</sup>, a member of the avian reticuloendotheliosis virus group. The vectors are replication defective and, to produce infectious particles, must obtain missing proteins in trans from packaging cells (10, 25). Once assembled in these cells, this virus is capable of infecting a wide variety of host cells. Since horizontal transmission of the virus is not possible in nonpackaging cells, only progeny of infected cells will express the proviral transcripts. The pCXIZ vector contains the SNV promoter as well as sequences required for encapsidation of viral transcripts, reverse transcription, and proviral integration (26). In addition, this vector contains an internal ribosome entry sequence followed by the bacterial *LacZ* gene. For efficient expression of this reporter gene in eukaryotic cells, a translation initiation sequence derived from the murine leukemia virus *gag* gene is present in the 5' flanking sequence. A cloning site is present upstream of the internal ribosome entry sequence region and was used for insertion of the  $\alpha 1(V)$  collagen cDNA. The pCXL vector is similar to pCXIZ, but lacks the internal ribosome-binding sequence (25). This vector was used as a control.

### Constructs

PCR splicing by overlap extension was used to add myc and signal sequences in frame to the  $\alpha 1(V)$  carboxyl terminus. The myc sequence was also obtained from Dr. Takashi Mikawa. The  $\alpha 1(V)$  signal sequence was obtained from the published chicken pro- $\alpha 1(V)$  sequence (20). To add the myc sequence, a 5' 93-mer was made that encoded a *SpeI* site, the myc sequence, and the sequence overlapping the 5' end of the  $\alpha 1(V)$  cDNA (5'TCGATCACTAGTGCTTGGGCGACCTCACCATGGAGC AAAAGTCCATTTCTGAAGAGGACTTGAATGCCCTTCCAGGATCCCCTGGCCAGATG 3'). A poly(T)<sub>30</sub> primer was used at the 3' end of the  $\alpha 1(V)$  cDNA, and PCR amplification generated a myc- $\alpha 1(V)$  cDNA. Digestion with *SpeI* produced a 1,244-bp fragment with a 3' end terminating just downstream of the stop codon.

The  $\alpha 1(V)$  signal sequence was added to the 5' end of this myc- $\alpha 1(V)$  cDNA fragment using a similar approach. Initially, the pro- $\alpha 1(V)$  signal sequence (and upstream translation initiation sequence) was PCR amplified using a 5' primer overlapping the signal sequence and possessing a *SpeI* 5' extension (5'TCGATCACTAGTGTGGACCATTGGATACGCACACGCGATG 3'), as well as a 3' primer encoding part of the signal sequence and an extension that would overlap with the myc sequence (5' CATGGTGAGGTCGCCAAGCAGAGGTCTGCAGGTTTCAGCTGC 3'). PCR amplification generated a 157-bp product encoding the signal sequence. This was annealed to the myc- $\alpha 1(V)$  cDNA and amplified by PCR using the same 5' primer and a 3' primer overlapping the stop codon and carrying a *SpeI* 3' extension (5'TCGATCACTAGTGGTGGCTCTTAGCCCATGAAG 3') to generate a 1,381-bp product. This  $\alpha 1(V)$  "minigene" was inserted into the *SpeI*-compatible *XbaI* site in the pCXIZ vector.

### Production of High Titer Recombinant Virus

The generation of infectious viral particles was achieved by transfection of packaging cells (NND17.2G) with the recombinant vectors using Lipofectamine (GIBCO BRL; 10). This packaging cell line was derived from a canine osteosarcoma (D17; 9, 40). A total of 2  $\mu$ g DNA comprised of recombinant vector and a pSV<sub>2</sub>-neo selection vector (in a 35:1 ratio) was used to transfect  $2 \times 10^4$  packaging cells in 30-mm dishes. Selection was achieved using cDMEM containing 400  $\mu$ g/ml (active units) Geneticin (G418) for a 2–3-wk period. Clones were isolated using cloning cylinders and expanded. Virus was harvested from confluent monolayers of packaging cell clones, and was frozen in small aliquots in liquid N<sub>2</sub>. To determine

1. Abbreviations used in this paper: SNV, spleen necrosis virus; TU, transducing units.

viral titers, chicken embryo fibroblasts were plated sparsely onto gridded 60-mm dishes. Aliquots of virus were used to infect the cells for 2–4 h in the presence of 10  $\mu$ g/ml polybrene (Sigma Immunochemicals, St. Louis, MO). 2 d after infection, the cells were fixed in 2% paraformaldehyde/PBS, and positive cells were identified using an assay for  $\beta$ -galactosidase activity (22). Frozen aliquots of virus were used at a concentration of 2–6  $\times 10^6$  transducing units (TU)/ml.

### Infection of Corneal Fibroblasts

Corneal fibroblasts (from first to fifth passage) were infected with  $\sim 5 \times 10^5$  TU of virus in the presence of 10  $\mu$ g/ml polybrene. Uninfected control cells also were cultured in the presence of polybrene. After infection for 5 h, media were changed and the cells cultured for at least 48 h until confluent.

### Cell Culture

Corneal fibroblasts from 14-d embryonic white leghorn chickens were isolated and cultured as described previously (23). Briefly, cells were isolated after bacterial collagenase treatment and were maintained in cDMEM containing 10% FBS (GIBCO BRL) and 50  $\mu$ g/ml ascorbate after the cells became confluent. Cells were trypsinized and split 1:2 or 1:3. Corneal fibroblasts also were cultured within three-dimensional bovine type I collagen gels (Vitrogen 100; Collagen Corp., Palo Alto, CA) as described previously (7, 8). Briefly, cells were trypsinized and counted, then placed in wells containing 0.5 ml of 0.75 mg/ml neutralized Vitrogen in complete media at a concentration of  $10^4$  cells per well. The collagen solution was polymerized at 37°C such that the cells were suspended in the gel. The edges of the collagen gels were released after overnight culture, allowing the gels to contract. Previously, we have extensively characterized this system for studies of collagen fibrillogenesis, and the newly assembled collagen fibrils are easily distinguishable from the bovine matrix (7, 8).

### Protein Analysis

Infected and uninfected 14-d chicken embryo corneal fibroblasts were cultured in monolayer cultures as described. At confluence, the fibroblasts were incubated for 4 h in cDMEM containing 1% FBS and ascorbate (50  $\mu$ g/ml). Universally labeled [ $^{14}$ C]proline (5  $\mu$ Ci/ml) was added, and the incubation was continued for an additional 20 h. Media were collected, the cell layer was rinsed twice with PBS, pooled with the media, and brought to 0.5 N acetic acid. Cell layers were scraped in 0.5 N acetic acid and brought to pH 2.5 with HCl. Media and cell layer were digested two times with pepsin (50  $\mu$ g/ml) over 24 h at 4°C. For electrophoretic analysis, loading was normalized to (a) constant pepsin-resistant cpm for analysis of collagen type I/V ratios; and (b) constant DNA content for analysis of type I levels. DNA was analyzed using Hoechst 33258 (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions and a TKO Fluorometer (Hoefer Scientific Instruments, San Francisco, CA). Samples were separated by electrophoresis on 6% SDS-polyacrylamide gels, dried, and exposed to BioMax film (Eastman Kodak, Rochester, NY). The films were scanned using the Eagle Eye II System (Stratagene, La Jolla, CA) and densitometric analysis was performed with Scanalytics software (ONE-Dscan, ver. 1.0; CSP, Inc., Billerica, MA). Quantities of types I and V collagen and the I/V ratio were determined by measuring the  $\alpha 2(I)$  and  $\alpha 1(V)$  bands, respectively.

### Northern Analyses

Total RNA was isolated from 14-d corneal fibroblasts infected with the  $\alpha 1(V)$  minigene-containing virus and control virus (containing only the *lacZ* sequence), and from uninfected cells. For each, confluent layers of fibroblasts grown in 100-mm dishes were used and infected with approximately  $5 \times 10^5$  TU of virus. RNA was isolated using Trizol (GIBCO BRL). Total RNA (5–10  $\mu$ g) was loaded and run on 1% formaldehyde-agarose gels. RNA was transferred to nylon filters (Hybond; Amersham, Arlington Heights, IL) and blots were probed with nick-translated cDNA fragments from  $\alpha 1(V)$  and  $\alpha 1(I)$  collagen, the retroviral vector, and G3PDH. Each Northern contained an RNA ladder (Promega, Madison, WI) as a size standard.

### Western Analysis for the Endogenous and Truncated $\alpha 1(V)$ Chains

To characterize the expression of the truncated protein, Western analysis

was performed as described above on a 6–20% gradient gel with a monoclonal anti-myc antibody (MYC1-9E10.2; 11). Infected and uninfected 14-d chicken embryo corneal fibroblasts were cultured for 24 h in cDMEM containing 1% FBS and ascorbate. Media was collected, and the cell layer was rinsed twice with PBS and pooled with the corresponding media. The cell layers were scraped in PBS. To determine whether the truncated protein was found in the cell layer, media, or both, comparable amounts of concentrated media and cell layer extracts were blotted onto nitrocellulose and probed either with a monoclonal anti-c-myc antibody to identify the truncated protein, or with a polyclonal anti- $\alpha 1(V)$  antibody to specifically identify the endogenous protein. Detection was by ECL chemiluminescence and exposure to BioMax film.

### Immunofluorescence Analysis of Truncated $\alpha 1(V)$ Protein

Infected and control corneal fibroblasts cultured on plastic or within collagen gels were fixed with 4% paraformaldehyde/PBS and methanol, quenched with 50 mM glycine/lysine, and blocked with 1% BSA/PBS. Cells were incubated for 1 h with anti-myc primary antibodies and followed with a second 1-h incubation with a fluorescein-conjugated secondary antibody. For localization of lysosomes, cells were incubated with rhodamine-conjugated dextran (Molecular Probes, Eugene, OR) at a concentration of 1 mg/ml for 2 h. The medium was changed, and the cells were viewed 24–48 h later. Cells were mounted in FITC Guard (Testog, Inc., Chicago, IL) and photographed.

### Transmission EM

Corneal fibroblasts in collagen gels were processed for transmission EM as described previously (7). Alternatively, collagen gel cultures were processed for preembedding immunoelectron microscopy as described previously (7). The following mAb were used: anti- $\beta$ -galactosidase (GIBCO BRL); anti-myc (myc1-9E10.2; 11); anti-type I collagen antibodies, I-BA1 and I-DD4 (17, 36); and an anti-NH<sub>2</sub> type V antibody (20). Sections were cut, stained with 2% aqueous uranyl acetate and 1% phosphotungstic, pH 3.2, and viewed with a transmission EM (CM10; Philips Technologies, Cheshire, CT).

### Analysis of Fibril Diameter

To determine the diameter of assembled fibrils in collagen gel cultures, fibrils that were adjacent to cells were selected. In addition, fibrils from immunolabeled sections and conventional plastic sections were analyzed. Diameters were measured from calibrated micrographs, and measurements were normalized using the internal 67-nm repeat of the fibrils.

## Results

### Construction of a Retroviral Construct Containing an $\alpha 1(V)$ Minigene

Reducing the endogenous  $\alpha 1(V)$  collagen levels using a dominant-negative retroviral strategy required the construction of an  $\alpha 1(V)$  minigene that would be capable of associating with endogenous  $\alpha$  chains to form hybrid molecules (strategy outlined in Fig. 1). We designed a truncated  $\alpha 1(V)$  minigene containing an  $\alpha 1(V)$  translation initiation and signal sequence that would direct synthesized protein to the secretory pathway, a myc peptide sequence to provide an epitope tag that could be used to identify mutant protein, and the carboxyl terminus of the  $\alpha 1(V)$  chain including a portion of the triple helix and the entire C-propeptide (Fig. 2 A).

To obtain the carboxyl terminal region of  $\alpha 1(V)$  collagen that is required for chain assembly, the carboxyl terminus of chicken  $\alpha 1(V)$  collagen was cloned using 3' RACE procedures. The coding sequence of the 1,360-bp cDNA that was obtained was verified using PCR cycle sequencing for both strands. The predicted amino acid se-

# DOMINANT-NEGATIVE STRATEGY

## Type V Retroviral Construct

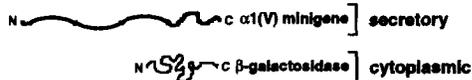


- LTR** Long Terminal Repeats
- Ψ** Packaging signal
- IRES** Internal Ribosome Entry Sequence
- lacZ** Gene encoding  $\beta$ -galactosidase

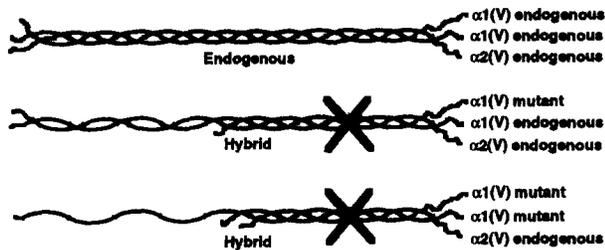
### Transcription



### Translation



### Chain Assembly



**Figure 1.** Targeted reduction of type V collagen using a dominant-negative approach. The dominant-negative strategy chosen to reduce the levels of type V collagen was designed to express high levels of a truncated  $\alpha$  chain that would compete with the endogenous  $\alpha$  chains for assembly into triple-helical molecules. The truncated  $\alpha$  chain is translated from what is effectively a polycistronic message containing the  $\alpha 1(V)$  minigene, an internal ribosome binding sequence, and a reporter gene encoding  $\beta$ -galactosidase. The truncated  $\alpha 1(V)$  sequence is directed to the secretory pathway for subsequent chain assembly, while  $\beta$ -galactosidase remains in the cytoplasm. The mutant-endogenous hybrid trimers formed are expected to be targeted to degradative pathways. With high expression of the mutant chains, the concentration of functional type V molecules, composed entirely of native  $\alpha$  chains, would be decreased.

quence from this clone has 390 residues from the carboxyl terminus and is composed of 267 non-triple-helical amino acids and 123 amino acids from the triple-helical domain (Fig. 2 B). This sequence has been submitted to GenBank/EMBL/DBJ and has the accession number U39621. To deliver this minigene to cells, the construct was inserted into SNV-based, replication-defective retroviral vectors. Retroviral particles containing this minigene have been designated "MVZ." Control virus carrying only the *LacZ* gene has been designated "CXL."

## Infection Rates for Recombinant Viral Particles Are High

To assess the infection rates for the recombinant viral particles, corneal fibroblasts were infected in vitro and assayed for cytoplasmic  $\beta$ -galactosidase expression. Virus (MVZ or CXL) was harvested from confluent monolayers of packaging cells and used to infect chicken corneal fibroblasts. The infection and culturing conditions we used yielded infection rates of 30–40% for the MVZ virus and  $\sim$ 40% for the CXL virus, demonstrating a high rate of infectivity for these cells.

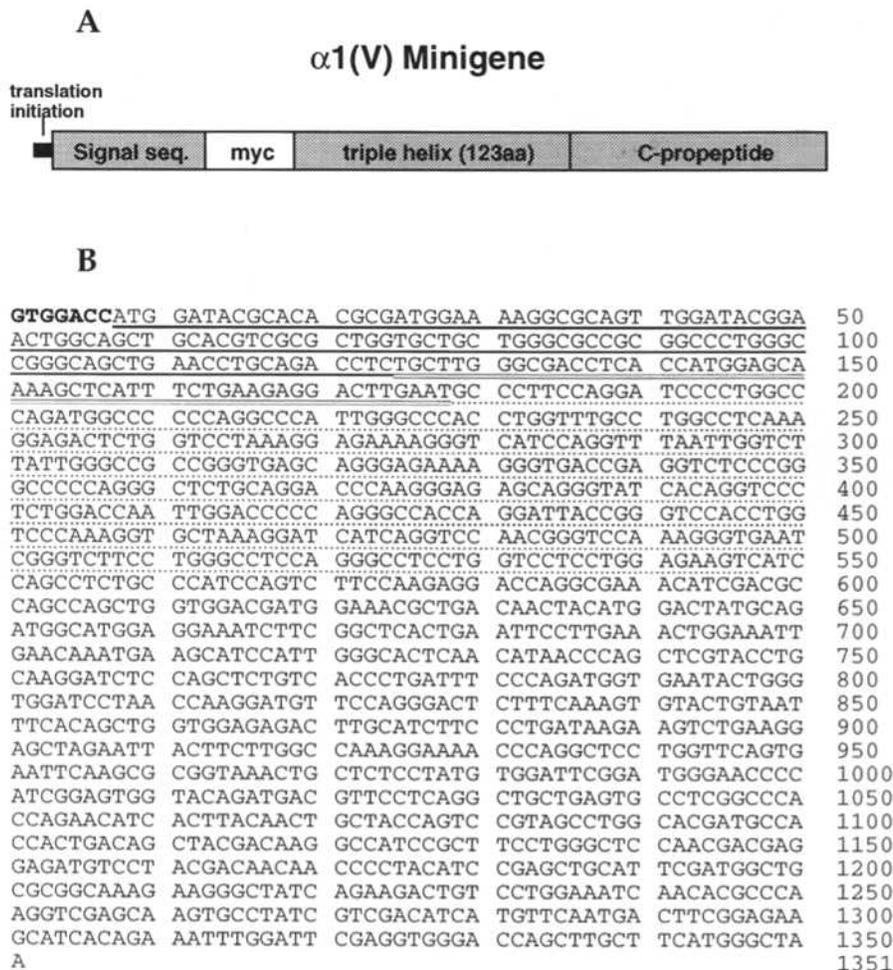
## Northern Analysis Demonstrates High Steady-state Levels of Retroviral Transcripts

To assay expression from the retroviral vectors, Northern analyses were performed (Fig. 3). Cells infected with the MVZ virus expressed high levels of the truncated  $\alpha 1(V)$  mRNA (5.6 kb) relative to the endogenous  $\alpha 1(V)$  mRNA (7.9 kb). This was not observed in CXL-infected or uninfected cultures. When probed with a 3'  $\alpha 1(V)$  cDNA (Fig. 3 A), hybridization was observed to both the truncated and endogenous  $\alpha 1(V)$  mRNAs, with the truncated form predominating. Although the endogenous  $\alpha 1(V)$  mRNA level appears high with this probe, this is caused by non-specific vector hybridization that comigrates with the endogenous  $\alpha 1(V)$  mRNA (see below). The  $\alpha 1(V)$  levels were more easily compared when a 5' probe specific for the endogenous mRNA was used (Fig. 3 B). Levels were relatively uniform in infected and control cultures, with the two virus-infected cultures showing a  $\sim$ 10% decrease relative to uninfected cultures. When blots were probed with the vector cDNA (Fig. 3 A), expression was high in both the CXL-infected and MVZ-infected cultures, with the size increase (4.1 to 5.6 kb) in the latter caused by the  $\alpha 1(V)$  minigene insertion. As described above, there is some slower migrating hybridization present in the viral-infected lanes but not in the uninfected lanes. The heterogeneity of this larger, nonspecific hybridization is most likely a result of the length and sequence differences that are present in the two mRNAs (with or without minigene), and is not observed in uninfected cells.

To determine whether viral infection influenced endogenous collagen expression, we analyzed  $\alpha 1(I)$  message levels in MVZ-infected and control cultures (Fig. 3 B). When probed with an  $\alpha 1(I)$  cDNA, mRNA levels in both the MVZ and CXL cultures were slightly reduced (10–15%) relative to the uninfected cultures. Some variability was observed, however. In five RNA preparations, the two extremes demonstrated both an increase and a decrease in  $\alpha 1(I)$  expression in MVZ infections relative to the controls. The other three preparations demonstrated the small but constant decrease (10–15%) in both MVZ- and CXL-infected cultures.

## Mutant $\alpha 1(V)$ Protein Is Expressed in MVZ-infected Cells

Western blot analyses using antibodies directed against the myc sequence included within the construct demonstrated the expression of a 54-kD protein specifically in the MVZ-infected cells (Fig. 4). The size is larger than the



**Figure 2.** Construction of an  $\alpha 1(V)$  minigene. The carboxyl terminus of the chicken  $\alpha 1(V)$  collagen was cloned and used to construct a truncated  $\alpha 1(V)$  minigene. The region of the cDNA used in the retroviral constructs contained 123 amino acids of the triple-helical domain and the entire 267-amino acid-long non-triple-helical C-propeptide domain. The  $\alpha 1(V)$  signal sequence and translation initiation sequence was added to the 5' end of the cDNA, and a myc sequence was added just downstream of this signal sequence (A). The sequence of the entire construct is presented in B. **Bold type**,  $\alpha 1(V)$  translation initiation sequence; **single underline**,  $\alpha 1(V)$  signal sequence; **double underline**, myc peptide sequence; **dotted underline**,  $\alpha 1(V)$  triple-helical region; **upper case**, entire  $\alpha 1(V)$  carboxyl propeptide sequence.

predicted 43 kD (49 kD with the signal peptide), and this can be attributed to posttranslational modifications (there are two potential sites for N-linked glycosylation) and to the altered electrophoretic mobility observed with collagens. The protein was present in the cell layer of the MVZ-infected, but not of the control-infected or uninfected cultures. Using dot-blot analyses, no protein was detected in the media of the cultures, even when concentrated, suggesting that the protein remains cytoplasmic (data not shown).

**Mutant  $\alpha 1(V)$  Protein Remains Intracellular and Is Localized to Lysosomes**

To determine the location of the mutant protein, infected and control cells cultured in three-dimensional collagen gels were examined by immunofluorescence using an antibody directed against the myc tag. In MVZ-infected cultures, positive cells showed reactivity throughout the cytoplasm (Fig. 5), frequently with a punctate distribution. As expected from the Western analysis, no signal was observed extracellularly in the collagen gel or in the CXL-infected or -uninfected cultures.

To determine if the truncated  $\alpha 1(V)$  protein was in the degradative pathway, double-labeling experiments were performed. Rhodamine-conjugated dextran was used as a lysosomal marker, and myc antibodies were used to localize the mutant  $\alpha 1(V)$  protein. The signals overlapped (Fig. 6),

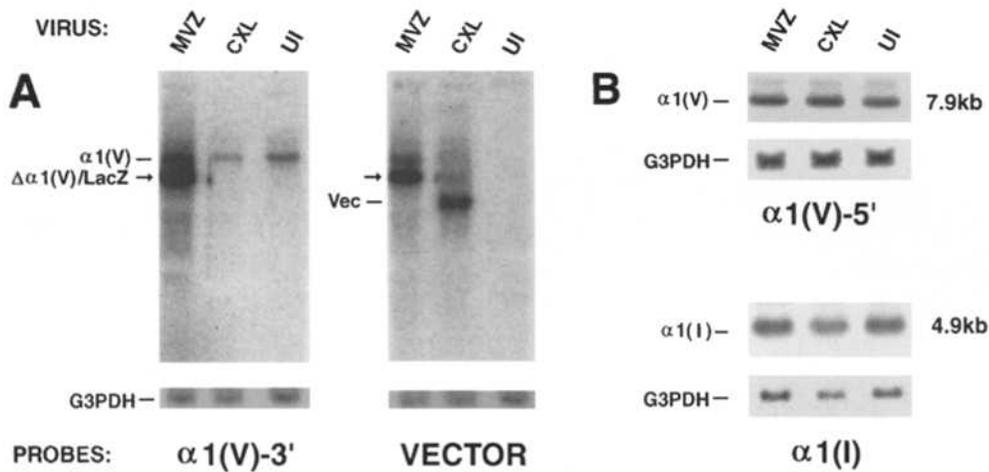
indicating that mutant proteins become localized to the lysosomal compartment within the infected cells. There was no signal for the mutant protein in the control cells.

**Type V Collagen Synthesis Is Reduced in Infected Cells**

Type V and type I collagen levels were measured in cells that had been infected with the MVZ virus, CXL control virus, or from uninfected cells. Infection rates were 40% for CXL and ~30% for the MVZ virus. The data demonstrate a ~25% reduction in type V collagen relative to type I collagen in the MVZ-infected cultures when compared to the control virus-infected and uninfected cultures (Fig. 7). Specifically, there were  $24.2 \pm 8.0$  and  $6.3 \pm 9.8\%$  (mean  $\pm$  SEM,  $n = 7$ ) reductions in the MVZ- and CXL-infected cultures, respectively. This indicates a specific reduction in type V collagen in cells expressing the type V minigene.

The decrease in the type I/V ratio seen in the MVZ-infected cells was further assessed by examining the absolute collagen levels. The  $\alpha 2(I)$  and  $\alpha 1(V)$  signals were measured in infected cultures and normalized to DNA content. A decrease of ~28% in the type I levels was observed in both virus-infected cultures (CXL and MVZ). The density values for the  $\alpha 2(I)$  chain for MVZ, CXL, and uninfected cultures were  $0.47 \pm 0.13$ ,  $0.47 \pm 0.07$ , and  $0.64 \pm 0.11$ , respectively (mean  $\pm$  SD,  $n = 6$ ). A decrease in type V collagen also was observed in both virus-infected cul-

## NORTHERN ANALYSIS OF INFECTED CORNEAL FIBROBLASTS



levels of the 5.6-kb truncated  $\alpha 1(V)$  mRNA (A, arrow labeled  $\Delta\alpha 1(V)/LacZ$  A). Infected and control cells all had comparable levels of endogenous  $\alpha 1(I)$  and  $\alpha 1(V)$  mRNAs (B) when normalized to a signal obtained from a G3PDH probe. When probed with the vector cDNA (A), expression was high in the MVZ-infected and CXL-infected cells, with the size increase in the former because of the  $\alpha 1(V)$  minigene insertion. No signal was observed in uninfected cells.

tures relative to the uninfected cultures. However, there was a further decrease in the MVZ-infected cultures resulting from expression of the minigene. This MVZ-specific effect resulted in the alterations observed in the type I/V ratio. The density values were  $0.26 \pm 0.03$ ,  $0.37 \pm 0.08$ , and  $0.42 \pm 0.08$  for MVZ, CXL, and uninfected cultures, respectively (mean  $\pm$  SD,  $n = 6$ ). Taken together, these data demonstrate a reduction of type V collagen relative to type I collagen, specifically in the MVZ-infected cultures, but not in the control virus-infected or -uninfected cultures.

### Collagen Fibril Morphology Is Altered in Cells Expressing the $\alpha 1(V)$ Minigene

To assess the effects of the  $\alpha 1(V)$  minigene expression on fibril assembly, collagen fibrils assembled by infected cells cultured in collagen gels were examined. Previous studies (7) have demonstrated that corneal fibroblasts cultured in three-dimensional collagen gels synthesize tissue-specific collagens and assemble fibrils with diameters comparable to those observed in situ. Fibroblasts isolated from 14-d

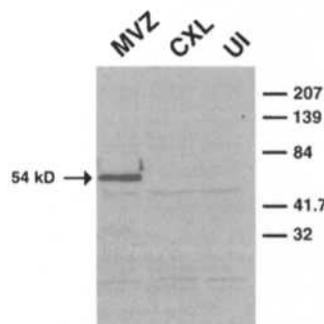


Figure 4. The truncated  $\alpha 1(V)$  protein is expressed in infected cells. Western blot analysis of MVZ- and CXL-infected and uninfected (UI) cell extracts demonstrates the presence of a protein with an apparent molecular mass of 54 kD specifically in the MVZ-infected extracts. This size is consistent with that predicted from the primary amino acid sequence

and likely posttranslational modifications. This band is not present in the control virus-infected or uninfected cell extracts. Western blots from reduced SDS-PAGE gradient gels (6–20%) were probed using an antibody directed against the myc tag present within the mutant  $\alpha 1(V)$  protein.

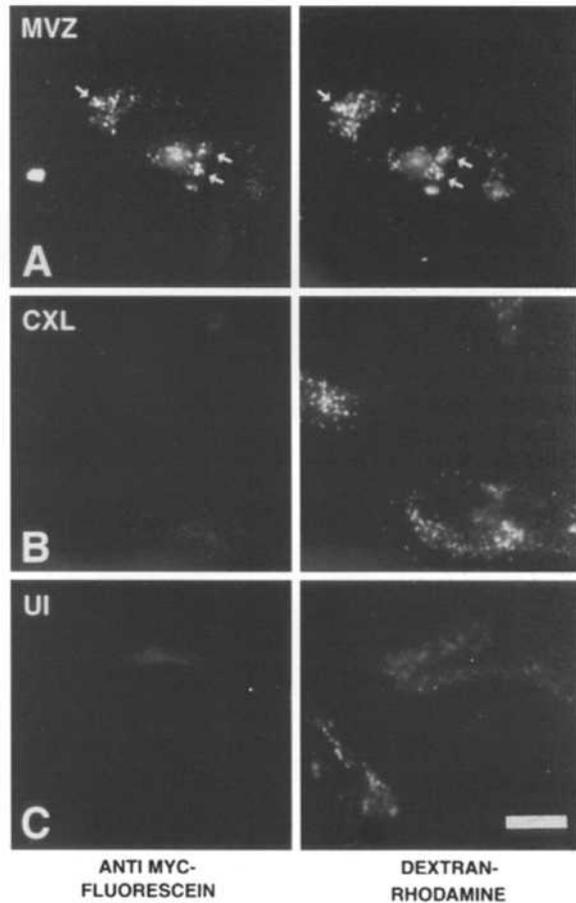
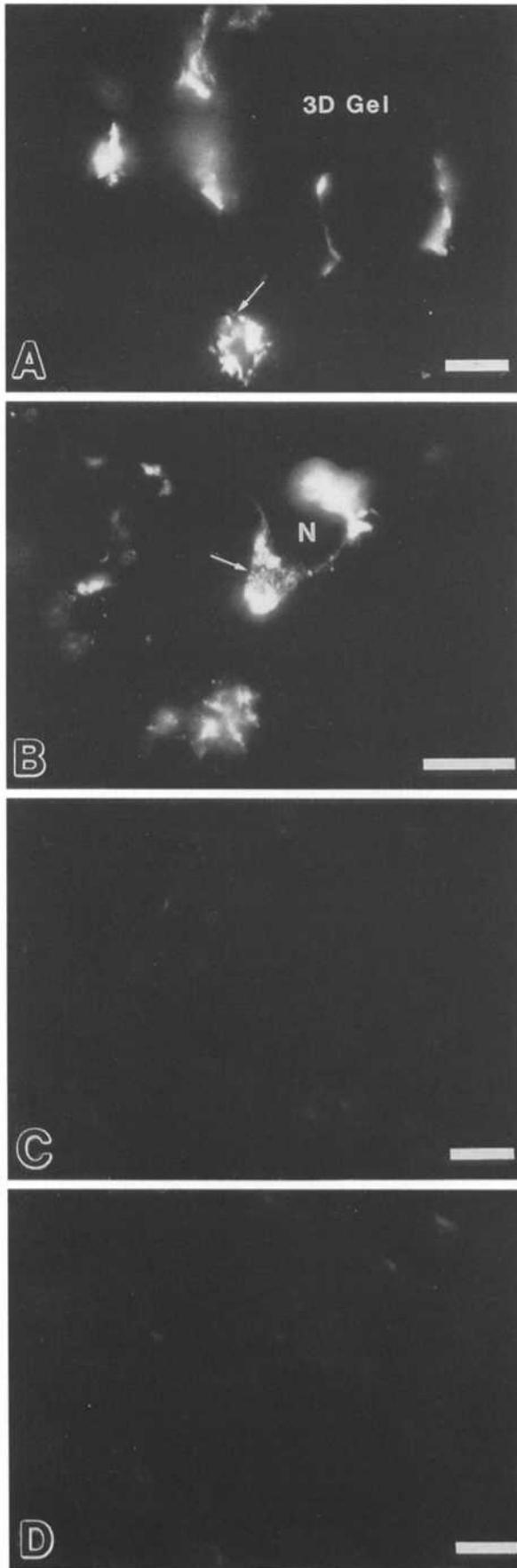
Figure 3. The  $\alpha 1(V)$  minigene expression is high compared to endogenous  $\alpha 1(V)$  collagen. Northern analysis of 14-d corneal fibroblasts infected with the MVZ virus, CXL control virus, and from uninfected cells (UI). Blots were probed with cDNA fragments encoding the  $\alpha 1(V)$  carboxyl terminus ( $\alpha 1(V)-3'$ ) and the vector sequence (A), and fragments encoding the  $\alpha 1(V)$  amino terminus ( $\alpha 1(V)-5'$ ) and  $\alpha 1(I)$  collagen (B). For normalization, all blots were probed with a G3PDH cDNA. Cells infected with the  $\alpha 1(V)$ -containing virus (MVZ) demonstrated high

corneas were infected with either the MVZ or CXL viruses and seeded into such three-dimensional gel cultures. Assays for  $\beta$ -galactosidase activity performed on parallel cultures indicated that infection rates were again  $\sim 30\%$ . This generated both infected and uninfected cell populations for morphological analyses.

To evaluate the effects of viral infection quantitatively, fibrils were measured from a number of infected cells cultured within the collagen gels. Infection was verified using anti- $\beta$ -galactosidase immunostaining, and measurements were made on fibrils that were immediately adjacent to the positive cells (Fig. 8). Control (CXL<sup>+</sup>) cells produced normal fibrils with respect to diameter and size distribution (mean = 24 nm), indicating that viral infection itself had no effect on fibril morphology. A total of 31 fibrils were measured from seven positive cells. The morphology of the fibrils synthesized by these cells was indistinguishable from the uninfected cells (data not shown). MVZ-infected cells (MVZ<sup>+</sup>), in contrast, produced large-diameter fibrils with a size distribution from 20 to 65 (mean = 37.2) nm. Data were collected from 14 cells and 62 fibrils were measured.

Since viral infection rates were 30%, it was possible to measure fibrils assembled by individual MVZ-infected and uninfected cells within the same culture. Uninfected (MVZ<sup>-</sup>) cells produced "normal" 24-nm fibrils, while the fibrils assembled by infected cells (MVZ<sup>+</sup>) produced large-diameter fibrils with a broad size distribution ranging from 20 to 60 nm (mean = 44 nm; Fig. 9). Several large diameter fibrils were visible in extracytoplasmic channels within these cells (2), clearly defining the cell producing them.

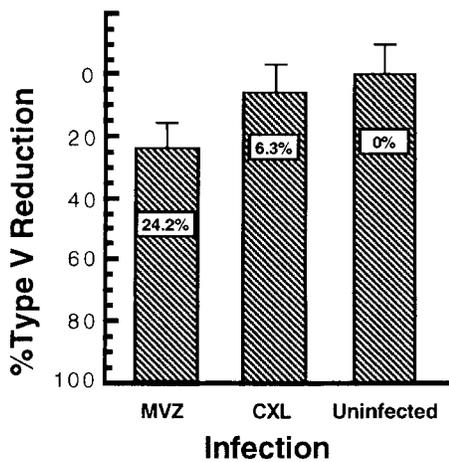
Previous studies in our laboratory have demonstrated that the amino-terminal domain of type V collagen, within heterotypic type I/V fibrils is exposed on the fibril surface. We therefore analyzed the infected cultures using immunoelectron microscopy with antibodies directed against this exposed domain. The small diameter fibrils showed significant reactivity for type V collagen while the large di-



**Figure 6.** Expression of the truncated  $\alpha 1(V)$  protein is localized to lysosomes. Double-label immunofluorescence was used to demonstrate that the mutant  $\alpha 1(V)$  protein is localized to the lysosomal compartment. Infected and control corneal fibroblasts were double labeled with rhodamine-conjugated dextran to identify the lysosomal compartment, and with anti-myc antibodies to identify the truncated  $\alpha 1(V)$  protein. The signals from the mutant  $\alpha 1(V)$  protein (anti-myc fluorescein, *left column*) and lysosomes (dextran-rhodamine, *right column*) were superimposable in MVZ-infected cultures. As expected, the CXL and uninfected cultures had no myc label, but the lysosomal compartment was labeled. Bar, 20  $\mu\text{m}$ .

ameter fibrils did not (Fig. 10). Both fibril populations were positive for chicken type I collagen (data not shown). These morphological data indicate that the content of type V collagen is reduced in fibrils assembled by the MVZ-infected cells, and that this reduction is sufficient to alter fibril assembly in such a way that abnormally large fibrils are deposited into the matrix.

**Figure 5.** The truncated  $\alpha 1(V)$  protein remains intracellular. Antibodies directed against the myc peptide tag were used to localize the mutant  $\alpha 1(V)$  protein in corneal fibroblasts cultured in bovine type I collagen gels. Immunoreactivity was strong in MVZ-infected cells (A and B), and was often observed in a punctate pattern within the cytoplasm (*arrows*). No signal was detectable in the extracellular matrix (*3-D Gel*). CXL control virus-infected and uninfected cultures (C and D) did not show any reactivity. Bars, 20  $\mu\text{m}$ .



**Figure 7.** Expression of type V collagen is reduced by infection with the  $\alpha 1(V)$  minigene. Cells infected with the MVZ virus synthesize reduced levels of type V collagen. Type V and type I collagen levels were measured from cultures that had been infected with the type V minigene-containing virus (MVZ), control virus (CXL), or from uninfected cultures. Type I/V ratios were measured by densitometric scans of the  $\alpha 2(I)$  and  $\alpha 1(V)$  bands. Levels of type V collagen have been normalized to the quantities of type I collagen. These data demonstrate that expression from the type V minigene-containing virus results in a 25% reduction of type V collagen relative to uninfected or control virus-infected cultures. Values are expressed as the mean  $\pm$  SEM,  $n = 7$ .

## Discussion

The regulation of collagen fibril assembly is critical to achieve tissue-specific matrix architecture. We have shown that reducing the type V collagen levels in type I/V fibrils abolishes this regulation and results in the assembly of fibrils with an abnormally large diameter. These data indicate that assembly of cornea-specific fibrils is an autoregulatory process. Fibrillogenesis is regulated by the components that comprise the fibril and, in this case, it is the quantitatively minor type V collagen within heterotypic fibrils that performs this regulatory function (3, 4, 19). Our previous studies *in vitro* implicated the concentration of type V collagen as being partially responsible for regulating fibril diameter (4). We also have shown that the corneal fibroblast produces a higher percentage of type V collagen (20%) than fibroblasts from other type I containing tissues, e.g., sclera and tendon (2–5%; 23). In addition, this is regulated by corneal fibroblast-specific cytoplasmic factors that can upregulate type V synthesis in noncorneal cell types (21). In the current studies, we demonstrate that the concentration of type V collagen alone is necessary and sufficient for tissue-specific fibril assembly.

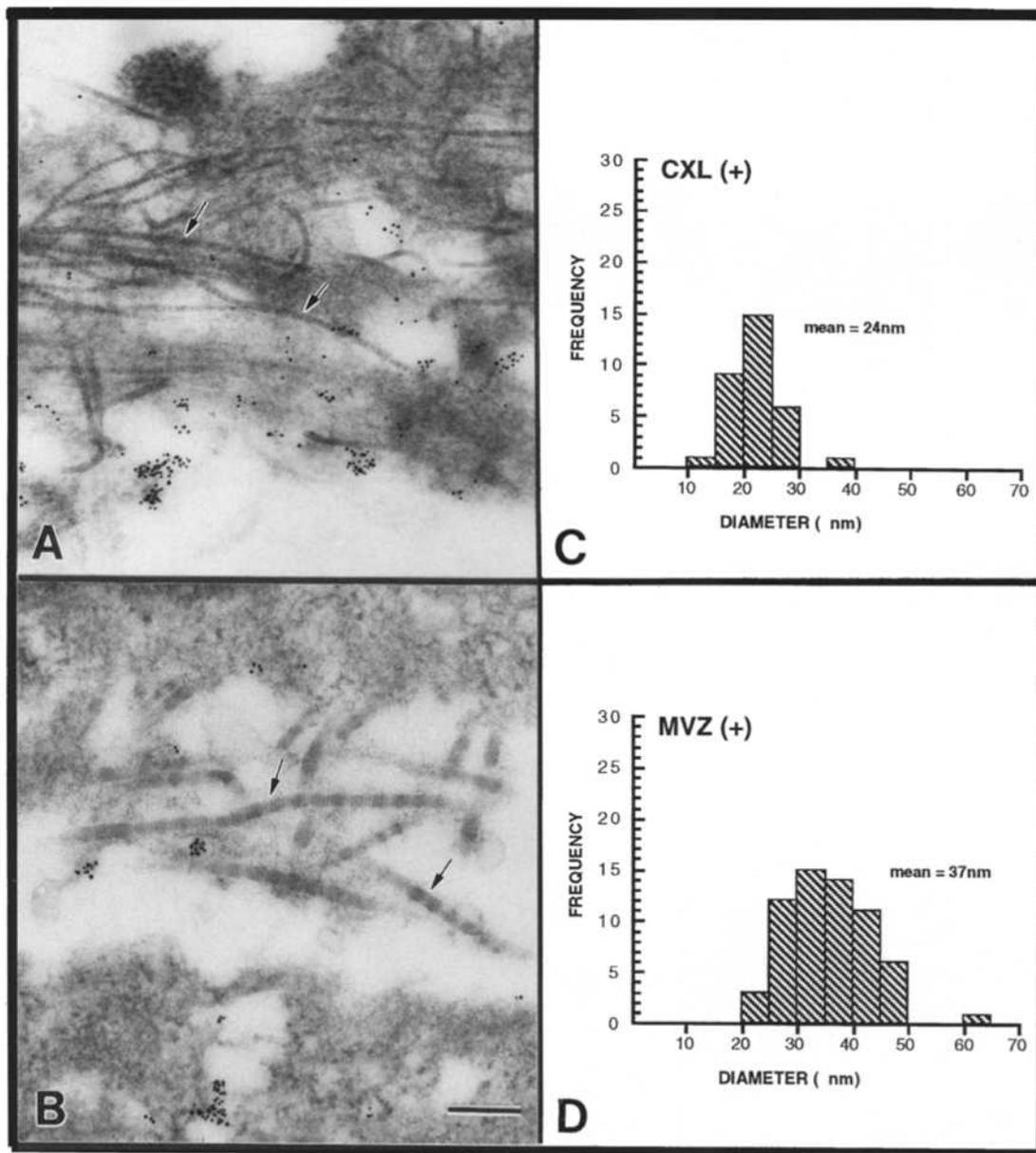
Other examples of this type of heterotypic fibril regulation exist. Types XI and II collagen, for example, coassemble as heterotypic fibrils in cartilage (24, 29) and loss of function mutations in the  $\alpha 1(XI)$  chain lead to the assembly of collagen fibrils with abnormally large diameters (16, 34). In addition, mutations that overexpress type II collagen result in a similarly altered XI/II ratio, and they also drive the assembly of large fibrils (13). Although it is likely that the  $\alpha 1(V)$  and  $\alpha 1(XI)$  chains have comparable regulatory functions since they are structurally very simi-

lar, comparisons between these  $\alpha$  chains are complicated by the fact that several alternatively spliced forms of type XI exist in different tissues (27, 32, 35, 37) and that heterotypic XI/II fibrils also may contain type IX collagen on their surface (24). It is likely that these modifications alter the properties of both the fibrils and their interactions with the surrounding extracellular matrix.

The dominant-negative strategy employed in the present study used a replication-defective retroviral vector to introduce the truncated  $\alpha 1(V)$  protein to cultured cells. Since infection rates were  $\sim 30\%$ , this allowed us to analyze infected cells in a wild-type background. We observed altered fibril morphology adjacent to infected cells, while the matrix synthesized by neighboring uninfected cells was normal. This demonstrates that the alteration of type V collagen levels is sufficient to abolish fibril diameter regulation, specifically in infected cells, and that compensatory mechanisms, if they exist, do not operate in the context of isolated mutant cells. This approach can be used to target specific tissues *in vivo* and would allow the analysis of tissue-specific effects without the complications that may arise from a systemic knockout.

Alternative approaches to alter collagen molecule concentration and/or function also have been used. Transgenic mutations, for example, have been made in the  $\alpha 2(V)$  chain in mice. The location of one such mutation encompasses the N-telopeptide and propeptidase cleavage site (1), and may prevent the formation of the “hinge” region that allows the amino-propeptide to project onto the fibril surface (20). Moreover, the molecules may be unable to participate in fibrillogenesis at all; the telopeptide domains removed as a result of the mutation are critical for normal chain assembly and fibrillogenesis (38). The homozygotes have numerous connective tissue defects in a variety of tissues, including fragile skin, alterations in hair follicle distribution, and corneal fibril defects, including an increase in diameter (1). These tissues have disparate type V concentrations, and thus it is likely that the wide range of connective tissue defects observed may be related to relatively small changes in type V concentrations. Recent evidence suggests mutations in  $\alpha 1(V)$  collagen in Ehlers-Danlos patients lead to morphological changes in tissues, such as skin, that have comparatively low type V concentrations (6). These results suggest important roles for type V collagen in matrix assembly in addition to the regulation of fibril diameter.

The strategy we used relies on the fact that collagen molecules assemble from the carboxyl termini of three  $\alpha$  chains. Expression of a truncated molecule that possesses a complete carboxyl terminus yet lacks the remaining sequences should result in the assembly of hybrid molecules that contain both truncated and endogenous  $\alpha$  chains. These hybrid molecules are likely to be shunted to degradative pathways, effectively lowering the concentration of type V collagen. The immunofluorescent localization of the truncated  $\alpha$  chains to the lysosomal compartment and the lack of extracellular signal are indicative of such intracellular degradation. Evidence in the literature also suggests that the assembled hybrid molecules are likely to be degraded. Several osteogenesis imperfecta variants have defects that delete large triple-helical portions of the affected  $\alpha$  chains but that leave the C-propeptide unaffected.



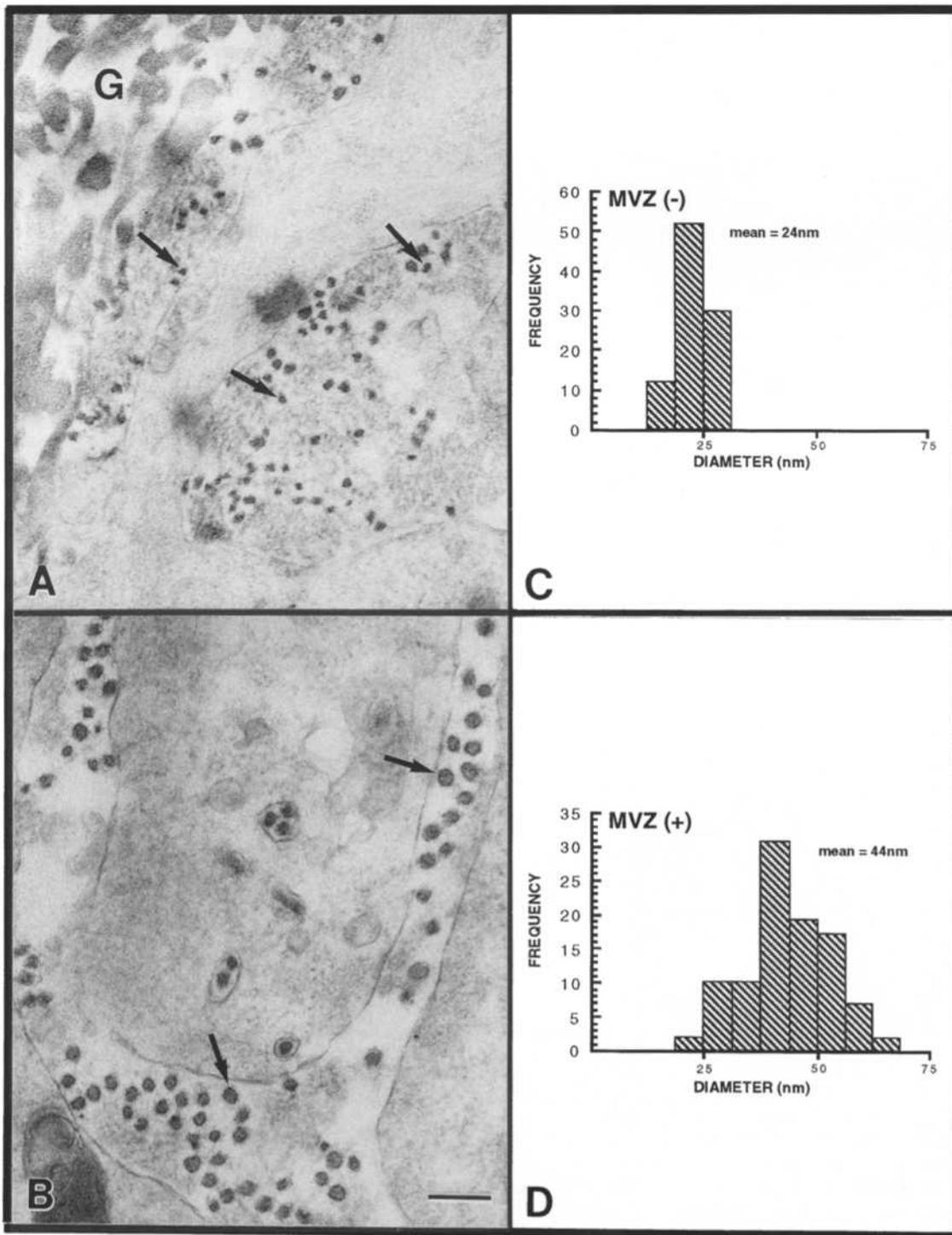
**Figure 8.** Cells expressing the  $\alpha 1(V)$  minigene assemble abnormally large diameter fibrils. Morphology of collagen fibrils assembled by MVZ- and CXL-infected corneal fibroblasts in three-dimensional collagen gel cultures. Immunostaining for cytoplasmic  $\beta$ -galactosidase was used to identify infected cells (gold particles in cytoplasm). CXL-infected cells (A and C) produced normal fibrils with respect to diameter and size distribution. A total of 31 fibrils were measured from 7 positive cells. The morphology of fibrils synthesized from CXL-negative cells was indistinguishable from the positive cells (not shown). MVZ-infected cells (B and D) produced large-diameter fibrils with a size distribution from 20 to 65 nm (mean 37.2 nm). Data were collected from 14 cells and 62 fibrils were measured. Bar, 150 nm.

In these cases, transcription is normal, but chain assembly is severely compromised and results in procollagen suicide (30, 41). The demonstration that endogenous  $\alpha 1(V)$  protein is reduced in MVZ-infected cultures indicates that the mutant  $\alpha$  chain is capable of this same *trans*-dominant effect on type V procollagen biosynthesis.

In the MVZ virus-infected cells, levels of the truncated  $\alpha 1(V)$  minigene transcripts are severalfold higher than the endogenous  $\alpha 1(V)$  mRNAs. Given that these truncated mRNAs share the same translation initiation and signal sequences as the endogenous mRNAs, they should compete effectively for translation and generate a high mu-

tant/endogenous protein ratio. Such high ratios could be sufficient to entirely block endogenous type V collagen synthesis in the infected cells by strongly favoring hybrid molecule formation over native chain assembly. This, in fact, is observed. The data comparing protein synthesis in infected and uninfected cells shows a 25% decrease in  $\alpha 1(V)$  protein in MVZ-infected cultures. Since only 30–40% of the synthesized matrix is the product of transgenic cells, this decrease reflects a significant reduction in the infected cells; the observed  $\alpha 1(V)$  protein may be solely the product of uninfected cells.

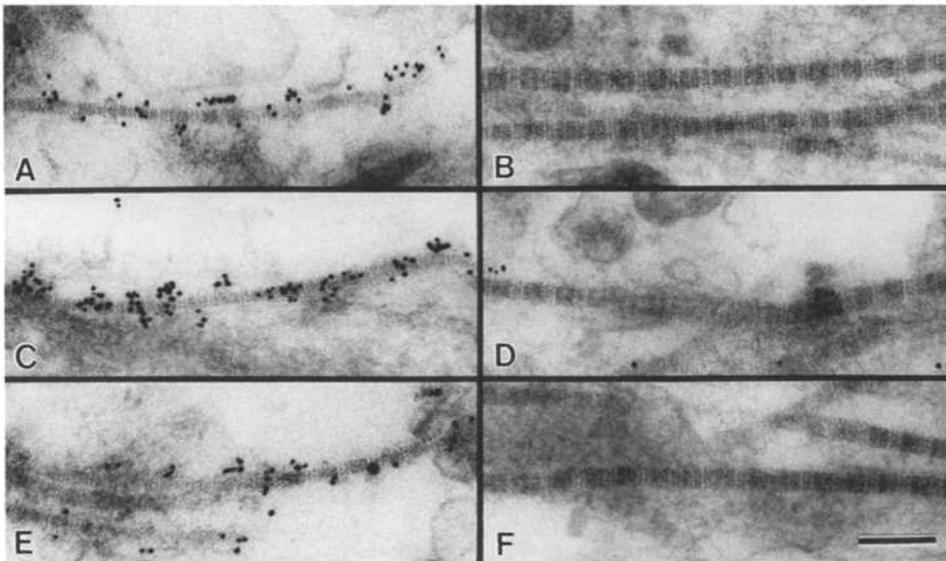
We observed nonspecific reductions in both type I and V



**Figure 9.** Cells expressing the  $\alpha 1(V)$  minigene produce large diameter fibrils while nonexpressing cells in the same culture produce small diameter fibrils. Collagen fibril morphology in MVZ-infected and uninfected chicken embryo fibroblasts cultured in collagen gels (G). Uninfected cells ( $MVZ^{-}$ ) produced normal 24-nm-diameter fibrils with a narrow size distribution (A and C). In MVZ-infected cells ( $MVZ^{+}$ ), the synthesized fibrils had a much broader size distribution, ranging from 20 to 60 nm with a mean of 44 nm (B and D). Several large diameter fibrils are visible in extracytoplasmic channels and the site of fibril assembly, and were seen as membrane delimited fibrils within the cytoplasm. Bar, 150 nm.

collagen synthesis as a result of the viral (CXL and MVZ) infection. These were observed at both the mRNA and protein levels. However, these reductions did not affect the ratio of types I and V collagen. This ratio was altered

only in the MVZ-infected cultures, and was therefore attributable to the expression of the type V minigene. The appearance of normal corneal fibrils in the CXL-infected cells indicates that these viral alterations do not result in



**Figure 10.** Immunoreactivity for type V collagen is inversely related to fibril diameter. Antibodies directed against the amino-terminal domain of type V collagen were used to localize this protein on fibrils assembled by the MVZ- and CXL-infected cells. Previous studies in our laboratory have demonstrated that this domain is exposed on the surface of heterotypic type I/V fibrils. Small-diameter fibrils produced by the CXL-infected cells demonstrated reactivity (A, C, and E) while the large fibrils assembled by the MVZ-infected cells were negative (B, D, and F). Bar, 150 nm.

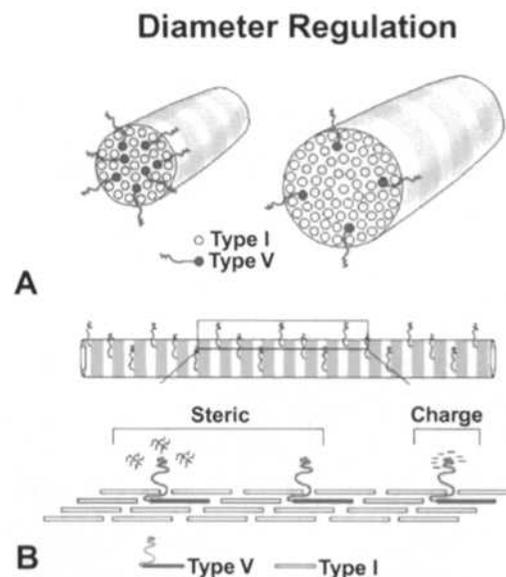
the loss of a differentiated phenotype; the alterations in fibril morphology and in the I/V ratios were only observed in the MVZ-infected cells.

Collagen fibril diameter is altered in cells infected with the MVZ virus. Analysis of fibrils assembled by the infected cells revealed that diameters were considerably larger than in control virus-infected or uninfected cultures. These fibrils also had a much broader size distribution than did the controls. The overall morphology and spacing of the fibrils assembled by the MVZ-infected cells is similar to that observed *in situ* in other tissues, e.g., tendon and sclera. In these tissues, the levels of type V collagen differ substantially from the levels found in the cornea. Type V collagen represents 15–20% of the fibrillar collagen in cornea, while it is only 2–5% in these other tissues (23). We have demonstrated that infection of corneal fibroblasts with recombinant virus lowers the endogenous type V collagen production. In these cells, the morphology of the fibrils synthesized resembles the morphology of fibrils synthesized by cells that normally produce comparably low type V collagen levels. This strongly indicates a direct relationship between type V collagen concentration and fibril diameter. Our demonstration that the large-diameter fibrils show little or no reactivity with anti-type V antibodies is consistent with this contention.

The dominant-negative strategy chosen to reduce the levels of type V collagen was designed to express high levels of a truncated  $\alpha$  chain that would compete effectively with the endogenous  $\alpha$  chains for assembly into triple-helical molecules. The mutant-endogenous hybrid trimers would be targeted to degradative pathways. With high expression of the mutant chains, the concentration of functional type V molecules, composed entirely of native  $\alpha$  chains, would be decreased. The altered fibril morphology we observed is most likely the result of the change in type I/V collagen ratios within the heterotypic fibrils that results from the lowered type V collagen available during assembly type I/V heterotypic fibrils. It is also possible that in addition to the mutant-endogenous hybrid formation, mutant homotrimers assemble as “mini collagens”. However, our immunofluorescent and Western analyses sug-

gest that if such molecules form, they are rapidly degraded or are accumulated in the cells, since no mutant protein was detectable by either assay in the extracellular matrix.

A model for the regulation of fibril diameter in heterotypic fibrils is presented in Fig. 11. We suggest that the amino-terminal domains of the type V collagen molecules



**Figure 11.** Model for type V regulation of collagen fibril diameter. Collagen fibrils within the cornea contain a high percentage of type V collagen and have small diameters (A, left), while fibrils in other tissues possessing low levels of type V collagen have large diameters (A, right). The experimental reduction of type V collagen described in this paper generates fibrils that have the characteristics of those found in tissues with low type V levels. A mechanism by which type V collagen may limit fibril growth is shown in B. Type V amino-terminal domains project onto the fibril surface, and when sufficient numbers have accumulated, they block further accretion of collagen monomers and thereby limit growth in diameter. The amino-terminal domain is large and possesses a number of acidic residues, and so may affect this block using steric and/or electrostatic hindrance.

are positioned at the fibril surface, providing a barrier to further accretion of collagen monomers. This would limit fibril growth by steric or electrostatic mechanisms. Steric mechanisms might involve interactions with other extracellular matrix components. Reduction of the type V molecules within the fibrils would eliminate this barrier and thus allow unhindered accretion and growth. Our data suggest that high levels of type V collagen are critical for the regulated assembly of small-diameter fibrils in the cornea, and that perturbation of this collagen is sufficient to lose this tissue-specific phenotype.

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