The abbreviations used are: EDS, Ehlers Danlos syndrome; pNCollagen, intermediate in the normal processing of type I procollagen to type I collagen containing the N-propeptides but not the C-propeptides; pNCollagen-"n", pNCollagen that lacks the 18-amino acid residues encoded by exon 6 in either COL1A1 or COL1A2 as a result of exon skipping (Weil et al., 1988, 1989, 1990; Vasan et al., 1991; Nicholls et al., 1991). In both genes exon 6 encodes the N-proteinase cleavage site and surrounding residues. Biochemical studies of tissues from one proband with EDS type VIIB showed that the abnormal and normal α2(1) chains occurred in almost equal amounts in the extracellular matrix of the skin and bone (Byers et al., 1985). Also, whereas in

EDS, a heterogeneous group of heritable disorders characterized by hypermobility of joints and abnormalities of skin, is classified into 11 types on the basis of clinical and biochemical findings (Beighton et al., 1988). EDS type VII is inherited in an autosomal dominant fashion and is distinct from other forms of EDS by virtue of marked joint hypermobility, multiple joint dislocations, and congenital hip dislocations that are usually bilateral (Byers, 1989). The biochemical basis of the disorder is a failure to process the N-propeptides of type I procollagen (for review see Byers, 1989).

Early studies of EDS type VII suggested that the impaired conversion of procollagen to collagen was the result of a deficiency of N-proteinase (Lichtenstein et al., 1973). EDS type VII was therefore thought to be the human counterpart of dermatosparaxis, a recessively inherited disorder of cattle (Lenars et al., 1971), cats (Counts et al., 1980; Holbrook et al., 1980), sheep (Fjølstad and Helle, 1974), and humans that is characterized by skin fragility and is caused by the absence of N-proteinase activity. However, in the five individuals with EDS type VII in whom the molecular defects are known, all were heterozygous for mutations in either the COL1A1 or COL1A2 collagen genes. The mutations resulted in the synthesis of proc chains that lacked the amino acid sequences encoded by exon 6 in either COL1A1 or COL1A2 as a result of exon skipping (Weil et al., 1988, 1989, 1990; Vasan et al., 1991; Nicholls et al., 1991). In both genes exon 6 encodes the N-proteinase cleavage site and surrounding residues. Biochemical studies of tissues from one proband with EDS type VIIB showed that the abnormal and normal α2(1) chains occurred in almost equal amounts in the extracellular matrix of the skin and bone (Byers et al., 1985). Also, whereas in
normal tissues the collagen fibrils are circular in cross-section, the fibrils in the skin and bone of that individual had rough borders and were near circular in cross-section (Eyre et al., 1985).

We show here that an individual with EDS type VII has a G to A transition at the 5' donor splice site of exon 6 in one of her alleles for the COL1A2 gene. Type I procollagen purified from the medium of the proband's dermal fibroblasts in culture and incubated with N-proteinase generated a mixture of pCcollagen and N-proteinase-resistant procollagen. Fibrils generated in vitro by cleavage of the mixture with C-proteinase (Kadler et al., 1987) initially formed hieroglyphic fibrils that could be resolved to fibrils with near circular cross-sections with additional N-proteinase. On the basis of our findings, we propose that the collagen fibrils in the tissues of individuals with EDS type VIIIB result from copolymerization of collagen and pCcollagen and partial cleavage of the abnormal pCcollagen by N-proteinase.

**EXPERIMENTAL PROCEDURES**

### Clinical History of the Patient and Her Family

The clinical details of the proband and members of her family have been previously described (Viljoen et al., 1987). The proband was the third child of a family where the mother and her four children had an inherited connective tissue disorder characterized by generalized articular laxity, joint dislocations and subluxations, and wormian bones in the skull. The latter feature may be common in EDS type VII but is not generally evaluated. A skin biopsy was taken from the proband for biochemical and molecular analyses. Dermal fibrils that grew from the biopsy in culture were used in the studies described here. Dermal fibrils from an unrelated healthy individual were used in control experiments.

### Source of Materials

Radiochemicals were from ICN Radiochemicals; sodium ascorbate was from Sigma; Dulbecco's modified Eagle's medium was from Northern Biologicals Ltd.; DEAE-cellulose was from Whatman; YM-50 ultrafiltration membranes were from Amicon; Sephacryl S-300 resin was from Pharmacia-LKB Ltd.; fertil egg eggs were from Northern Biological Supplies; spectrophotometrically pure carbon (rods) were from Agar Aids; copper grids were from Gilder Grids; Sequenase was from United States Biochemicals; water used in the preparation and storage of samples (see the legend to Fig. 2) was from a commercial water purification system that comprised tap water feeding into a Millipore R06 Plus cartridge pack (Millipore) connected in-line to a Millipore Milli-Q Plus UltraPure water purification for final delivery.

### DNA Sequence Determination

Total RNA was prepared from cultured dermal fibroblasts (Chromogynski and Sacchi, 1987; Greenberg, 1987). Ten μg of RNA were precipitated with 1 μg of a Saff-tailed oligonucleotide primer, A, complementary to coding sequence in exons 8 and 9 of COL1A2 (sequence 5': TACGTCGACGTCCGGGTTTCCAGGGTG). The cDNA was prepared as described elsewhere (Maniatis et al., 1982; Willing et al., 1990). An EcoRI-tailed primer, B, identical to coding sequence in exons 2 and 3 (sequence 5': GCGAAATTCGTACGAGGAACTCTCAG) and primer A were used to amplify cDNA synthesized from the COL1A2 gene spanning exon 6 using the polymerase chain reaction (Saiki et al., 1988). The amplified cDNA fragment was cloned into M13 mp19. Single-stranded DNA was prepared (Messing et al., 1981) and sequenced by the dye deoxy chain termination method (Sanger et al., 1977) with 77 polymerase Sequenase*. Genomic DNA was prepared from the patient's dermal fibroblasts in culture using standard procedures. The genomic DNA sequence spanning exon 6 and including the intron 5 acceptor and intron 6 donor splice sites was amplified using an EcoRI-tailed primer, C, within intron 5 sequence 5': ATAGAATTCGGTCTAAGCATTACGTAACA and a Saff-tailed primer, D, within intron 6 sequence 5': CAGCACATTTATTTAGCTACCTAAGTTA). The amplified fragment was cloned and sequenced as above.

### Preparation of Procollagen

1C-Labeled type I procollagen was purified from the culture medium of normal and proband dermal fibroblasts (passage 7-10) using the methods described previously (Kadler et al., 1987). In brief, fibroblasts were grown to confluence and incubated in Dulbecco's modified Eagle's medium supplemented with 1 μCi/ml of a mixture of uniformly labeled C-14-amino-acids, 25 μg/ml ascorbic acid, and no serum. Proteins in the culture medium were precipitated by ammonium sulfate, and the type I procollagen was chromatographed on two consecutive columns of DEAE-cellulose (Fiedler-Nagy et al., 1981; Feltenon et al., 1980). The procollagen was concentrated by ultrafiltration and stored at -20 °C in storage buffer consisting of 0.1 M Tris-HCl buffer (pH 7.4 at 20 °C) containing 0.4 M NaCl and 0.01% NaN3. Procollagen concentration was determined by a colorimetric hydroxyproline procedure (Woessner, 1961), assuming 10.1% hydroxyproline by weight procollagen (Fiedler-Nagy et al., 1981). The procollagens had a specific radioactivity of 1500 cpm/μg.

### Procollagen N- and C-proteinases

The C-proteinase was purified from the culture medium of the leg tendons of 250 dozen 17-day chick embryos as described previously (Hojima et al., 1985). The preparation had 400 units/ml activity where 1 unit is the amount required to cleave 1 μg of type I procollagen/h at 34 °C in a reaction system containing procollagen at a concentration of 10 μg/ml and 0.001 M Tris-HCl buffer (pH 7.4 at 20 °C) containing 0.15 M NaCl, 5 mM CaCl2, 0.01% NaN3, and 0.01% Brij (35). The reaction was stopped by the addition of 0.1 volume of 1 M Tris buffer (pH 7.4 at 20 °C) containing 0.25 M EDTA and 0.1% NaN3. 0.2 volume of 50% sucrose was added and the pCcollagen was isolated by Sephacryl S-300 gel filtration as described previously (Kadler et al., 1987). The pCcollagen was concentrated by ultrafiltration and stored in storage buffer at -20 °C. In subsequent experiments that examined the effects of partial cleavage of the abnormal procollagen, the mixture of pCcollagen and procollagen (6 μg) under the same conditions as for control samples (6 μl, 8 h, 34 °C), the reaction was stopped by the addition of Tris-EDTA, and the resultant mixture of pCcollagen and uncleaved procollagen was purified with Sephacryl S-300 gel filtration. The preparation had 800 units/ml activity.

### Preparation of Substrates for Fibril Formation

For control experiments, pCcollagen was generated by incubating type I procollagen (2500 μg) with partially purified N-proteinase (140 units) in a volume of 6 ml for 8 h at 34 °C in 0.05 M Tris-HCl buffer (pH 7.4 at 20 °C) containing 0.15 M NaCl, 5 mM CaCl2, 0.01% NaN3, and 0.01% Brij (35). The reaction was stopped by the addition of 0.1 volume of 1 M Tris buffer (pH 7.4 at 20 °C) containing 0.25 M EDTA and 0.1% NaN3. The mixture was concentrated by ultrafiltration and stored in storage buffer at -20 °C. In subsequent experiments that examined the effects of partial cleavage of the abnormal procollagen, the mixture of pCcollagen and procollagen (6 μg) was incubated with N-proteinase (12 units) in a final volume of 83 μl. The reaction was stopped by the addition of 25 μl of 81% ethanol (-20 °C). The proteins were collected by centrifugation at 15,000 X g for 15 min (at 4 °C) and dried in a flow of dry nitrogen. The mixture pCcollagen/partially cleaved procollagen was resuspended in fibril formation buffer (see below) and used directly in experiments.

### Analysis of N-proteinase Cleavage

Products of N-proteinase cleavage were separated by SDS-PAGE using 7% separating and 3.5% stacking gels according to Laemmli (1970). The gels were visualized by fluorography. Fluorograms were prepared by equilibrating gels in 20% diphenyloxazole in glacial acetic acid and exposing dried gels to prefilled Kodak X-Omat AR film at -70 °C. Fluorograms of the pellet fractions of fibrils were analyzed by laser densitometry and the amounts of collagen and pNcollagen molecules in fibrils were calculated, after correction for molecular mass, from the relative intensities of the a2(I) and pN2I(II)* bands, respectively.

### Fibril Formation

The substrates (pCcollagen in control samples and the mixture pCcollagen/procollagen in the EDS type VIIIB samples) and C-proteinase were dialyzed separately against 2 × 600 volumes of fibrin forma-
tion buffer consisting of 20 mM NaHCO₃, 117 mM NaCl, 3.4 mM KCl, 1.8 mM CaCl₂, 0.81 mM MgSO₄, 1.03 mM NaH₂PO₄, and 0.01% NaN₃ (pH 7.4 at 20 °C). Fibril formation was initiated by mixing the substrate (100 μg/ml) and C-proteinase (50 units/ml) in a 1.5-ml microcentrifuge tube, and the tube was incubated at 37 °C for 24 h. To prevent changes in pH and volume of the solutions, the tube was gassed with water-saturated air, and the tube was incubated at 37 °C for 24 h.

To examine the composition of the fibrils formed, the reaction mixture was centrifuged at 15,000 x g for 5 min, and the pellet and supernatant fractions were analyzed separately by SDS-PAGE and fluorography as described above.

Electron Microscopy of Thin Cross-sections of the Fibrils

Fibrils were collected by centrifugation and secured in a drop of warm 0.1% agar to facilitate handling. The fibrils were fixed in 4% formaldehyde, stained with 1% phosphotungstic acid and 1% uranyl acetate (pH 4.4), and embedded in an araldite epoxy resin. Thin sections were obtained using a diamond knife and a LKB-ultracut ultramicrotome. The sections were post-stained in 1% phosphotungstic acid and 1% uranyl acetate (pH 4.4) and examined in the JEOL 1200EX transmission electron microscope operated in the conventional mode. Images were recorded on Ilford E.M. film.

Scanning Transmission Electron Microscopy (STEM)

(i) Sample Preparation—Carbon films were prepared by evaporation onto freshly cleaved mica, using carbon rods as a source. Carbon film thickness was typically 2-2.5 nm, as measured by electron scanning procedures for a STEM. The films were floated onto a clean water surface and collected on 400-mesh copper grids that had previously been ultrasonically cleaned for 15 min in acetone. A drop of the sample was placed on a filmed grid, and the sample was allowed to adsorb for 15 s. The grid was flushed with six drops of water and allowed to air dry.

(ii) Use of a Conventional Instrument in the STEM Mode—The basic instrument was a JEOL 1200EX transmission electron microscope equipped with a JEOL ASID10 scanning unit and the standard lens settings. The collection angle of the annular dark-field detector ranged from 25 x to 75 x radians; the effective detector size was 8.5 nm. The STEM was operated at 120 kV with the standard instrumental magnification setting of X25,000 used here, the pixel size was 8.5 nm. The signal from the dark-field detector was linearly dependent on carbon film mass thickness up to approximately 50 kDa nm⁻².

(iii) Measurement of Transverse Mass Distributions—Mass mapping procedures were similar to those developed for a dedicated STEM instrument with a field emission gun (Engel, 1978, 1982; Engel et al., 1981; Engel and Reichelt, 1984; Freeman and Leonard, 1981). Micrographs were acquired using an ~3-nm spot size and exposing the specimen to a low electron dose (~10⁶ electrons nm⁻²). A diffraction grating replica (2160 lines/mm) was used for magnification calibration and was estimated to be accurate to better than 2%. At the instrumental magnification setting of X25,000 used here, the pixel size was 8.5 nm. Images were at room temperature during electron microscopy. Image analysis routines for obtaining mass distributions of fibrils were written in the SEMPER 5 image analysis program (Synoptics Ltd., Cambridge, United Kingdom).

RESULTS

Mutation Analysis—Using the methods described by Cohn and Byers (1990) for the routine screening of the type I collagen synthesized by dermal fibroblasts, 50% of the patient's procollagen was found to be resistant to cleavage by pepsin at the N-terminal end and 50% was cleaved to α2(I). In control samples all the procollagen chains were converted to α2(I) chains (results not shown). The pattern of cleavage in the proband's samples was like that observed for patients with EDS type VIIb (Cohn and Byers, 1990). cDNA clones spanning exon 6 of COL1A2 were prepared from the proband's RNA and sequenced. Half of the clones lacked precisely the sequences encoded by exon 6 (Fig. 1A). On sequencing the genomic DNA clones spanning exon 6, a G to A transition was found at the first position in the consensus donor splice site of intron 6 (arrow) leads to the deletion of exon 6 in the COL1A2 cDNA. C, DNA and protein sequence of exon 6 and partial sequence of introns 5 and 6. The arrow marks the site of the mutation.

FIG. 1. Sequence of the mutation, cDNA sequence (A) and genomic DNA sequence (B) of the normal and the mutant COL1A2 allele from the proband with EDS type VII. The G to A transition in the donor splice site of intron 6 (arrow) leads to the deletion of exon 6 in the COL1A2 cDNA. C, DNA and protein sequence of exon 6 and partial sequence of introns 5 and 6. The arrow marks the site of the mutation.

FIG. 2. N-proteinase cleavage of type I procollagen from control and the EDS type VII cells. One-μg type I procollagen was incubated at 34 °C with 1 unit of N-proteinase in control samples and 3 units of N-proteinase in EDS type VII samples. Lane 1, control procollagen non-incubated; lanes 2 and 3, control procollagen incubated for 8 h; lane 4, patient procollagen non-incubated; lanes 5 and 6, patient procollagen incubated for 8 h. Further processing of the chains to α1(I) and α2(I) chains was observed in some of the control samples.

Cleavage of Control and Proband Type I Procollagen with N-proteinase—To examine the consequences of the mutation on processing of the procollagen by N-proteinase, type I procollagen was purified from the medium of control and EDS type VII dermal fibroblasts. The procollagens were incubated with N-proteinase and the reaction products were examined by SDS-PAGE and fluorography (Fig. 2). No differences in migration were observed between control and EDS type VII procollagens (compare lanes 1 and 4, Fig. 2). After treatment
with N-proteinase all the \( \alpha_1(I) \) and \( \alpha_2(I) \) chains were converted to \( \alpha_1(1) \) and \( \alpha_2(1) \) chains in control samples (Fig. 2, lanes 2 and 3). In the EDS type VII samples, 50% of the \( \alpha_1(1) \) chains and 50% of the \( \alpha_2(1) \) chains were converted to \( \alpha_1(1) \) and \( \alpha_2(1) \) chains, respectively (Fig. 2, lanes 5 and 6). Thus, the absence of the N-proteinase cleavage site in the \( \alpha_2(1) \) chains conferred resistance to cleavage by N-proteinase to the \( \alpha_1(1) \) chains in the same molecules.

**Copolymerization of Collagen and the N-proteinase-resistant \( \alpha_1 \) (Ncollagen–)– Fibris were formed by incubating the mixture of \( \alpha_1 \) (Ncollagen)/N-proteinase-resistant procollagen with C-proteinase. Similarly, fibrils were generated in control samples by incubating \( \alpha_1 \) (Ncollagen) with C-proteinase (Kadler et al., 1987, 1988, 1990a). The fibrils formed from control samples were circular in cross-section, cross-striated, and were like those generated previously in the fibril-forming system (Kadler et al., 1990b) (Figs. 3A and 4A). Fibris formed from the EDS type VII samples were highly irregular in cross-section, cross-striated, and resembled the hieroglyphic fibrils seen in the skin of dermatosparactic animals and man (Figs. 3B and 4B). We noted that the circularity of fibrils generated in control samples was often difficult to assess because the fibrils tended to clump and coalesce. This was attributed to the forces imparted on the fibrils by the centrifugation step used to collect the fibrils. A gentler method of preparing fibrils for electron microscopy, and consequently a more reproducible way of accessing their circularity, was to deposit the fibrils on carbon-coated electron microscope grids and to examine the distribution of mass across the fibril (the transverse mass distribution) using STEM. An additional feature of STEM was that the transverse mass distribution along the entire length of the 20–100 fibrils collected/grid could be examined. The analyses showed that the transverse mass distributions of all the fibrils generated in control samples were symmetrical and that the fibrils were uniform diameter cylinders (Fig. 4A). No fibrils were observed that did not have symmetrical, uniform transverse mass distributions. In contrast, the transverse mass distributions of all the fibrils generated in the EDS type VII samples were asymmetric (Fig. 4B), and the fibrils exhibited a marked deviation from circularity. The STEM results were in good agreement with the hieroglyphic patterns of the fibrils observed in cross-section (Fig. 3B). The STEM analyses also showed that the fibrils were as long and apparently as flexible as those generated in control samples.

To examine the molecular composition of the hieroglyphic fibrils, fibrils were collected by brief centrifugation and the pellet (fibril) and supernatant (soluble) fractions were analyzed separately by SDS-PAGE and fluorography (Fig. 5). In control samples, the pellet fractions contained \( \alpha_1(1) \) and \( \alpha_2(1) \) chains, and the supernatant fractions contained the cleaved C-propeptides and small amounts of \( \alpha_1(1) \) and \( \alpha_2(1) \) chains that were only visible after long exposures of the gels to film (data not shown). The \( \alpha_1(1) \) and \( \alpha_2(1) \) chains in the supernatant were from the critical concentration of collagen in equilibrium with fibrils (Kadler et al., 1987; Na et al., 1989).
pNcollagen—Fibrils formed from collagen and partially cleaved pNcollagen molecules. Filaments were formed as described in Fig. 1, lane 1, pNtr1(I), pNn2(I), pNal(I), and pNn2(I)'''' chains. Quantitation of the pellet fraction of the hieroglyphic fibrils contained control filaments; pNtr1(I), pNn2(I)'''' chains were not detected (Eyre et al., 1985). To test the hypothesis that absence of pNtr1(I) chains were part of molecules in which some pNn2(I)'''' chains had been cleaved to crl(I) chains. Fibrils formed from these molecules differed from the hieroglyphic patterns seen previously in that they had symmetrical transverse mass distributions (Fig. 4C) and were therefore uniform cylinders.

Copolymerization of Collagen and Partially Cleaved pNcollagen—Fibrils formed from collagen and partially cleaved pNcollagen molecules contained a1(I), a2(I), pNn2(I)'''' chains, and small amounts of pNn1(I) chains (Fig. 7, lane 1). Quantitation of a1(I), a2(I), pNn1(I), and pNn2(I)'''' chains in fibrils (by laser densitometry of fluorograms and correction for molecular mass) showed that the ratio a2(I)/pNn2(I)'''' chains in fibrils was approximately 4:1. Only small amounts of pNn1(I) chains were detected. Consequently, the fibrils comprised approximately 80% collagen, approximately 20% pNcollagen molecules in which both the pNn1(I) chains were cleaved to a1(I) chains, and trace amounts of pNcollagen molecules in which one or both of the pNn1(I) chains remained intact. The analyses also showed that the amounts of a1(I) chains were equal to twice the sum of the amounts of the a2(I) and pNn2(I)'''' chains. These data indicated that the pNn2(I)'''' chains were part of molecules in which some pNn1(I) chains had been cleaved to a1(I) chains. Fibrils formed from these molecules differed from the hieroglyphic patterns seen previously in that they had symmetrical transverse mass distributions (Fig. 4C) and were therefore uniform cylinders.

The supernatant fraction of the fibrils contained predominantly a1(I) chains and also pNn1(I) and pNn2(I)'''' chains. Only small amounts of a2(I) chains were detected (Fig. 7, lane 2). The relative proportions of the chains in the supernatant were consistent with the presence of pNcollagen molecules in which one of the pNn1(I) chains

Fig. 5. Molecules contained within normal and hieroglyphic fibrils. Fibrils were formed as described in Fig. 3, collected by brief centrifugation, and the molecules in the pellet and supernatant fractions were examined separately. Lane 1, pellet fraction of control fibrils; lane 2, supernatant fraction of control fibrils; lane 3, pellet fraction of EDS type VII fibrils; lane 4, supernatant fraction of EDS type VII fibrils.

The pellet fraction of the hieroglyphic fibrils contained pNtr1(I), pNn2(I)'''' chains. Quantity of the amounts of a2(I) and pNn2(I)'''' in fibrils by laser densitometry of fluorograms and correction for molecular mass, showed that pNcollagen' accounted for 52 ± 6% S.D. (n = 3) of the protein in the fibrils. Collagen accounted for 48% of the protein. The supernatant fraction of the fibrils contained the cleaved C-propeptides and pNtr1(I), pNn2(I)'''' with small amounts of a1(I) and a2(I) chains.

Cleavage of the proa1(I) Chains in Abnormal Procollagen Molecules by Elevated Amounts of N-proteinase—Extracts of skin and bone from individuals with EDS type VIIR contain expected of pNtr1(I), pNn2(I)'''' chains were not detected (Eyre et al., 1987). To test the hypothesis that absence of pNn1(I) chains in extracts of EDS type VIIB tissues was the result of N-proteinase activity, the abnormal type I procollagen was incubated with the highest concentration of N-proteinase in the presence of 25 mM EDTA (lane 3, procollagen treated with N-proteinase; lane 4, procollagen incubated at 34 °C for 8 h in the absence of N-proteinase.

Fig. 6. Partial cleavage of the abnormal procollagen by treatment with elevated levels of N-proteinase. The EDS type VII procollagen (2 µg) was incubated with N-proteinase (145 units/ml) at 34 °C for 8 h. Lane 1, unincubated procollagen; lane 2, procollagen treated with N-proteinase; lane 3, procollagen treated with N-proteinase in the presence of 25 mM EDTA; lane 4, procollagen incubated at 34 °C for 8 h in the absence of N-proteinase.
were converted to \( \alpha_1(I) \) chains. The virtual absence of \( \alpha_2(I) \) chains indicated that collagen represented a very small fraction of the soluble molecules. Molecules containing \( \mathrm{pN}2(\mathrm{I})^{\text{ext}} \) and cleaved \( \mathrm{pNa}1(\mathrm{I}) \) chains were not incorporated into fibrils as readily as molecules in which no cleavage had occurred (compare Fig. 7, lane 1, and Fig. 5, lane 3).

**N-proteinase Treatment of Hieroglyphic Fibrils**—Although the fibrils formed by copolymerization of collagen and \( \mathrm{pNcollagen}^{\text{ext}} \) had near circular cross-sections, they differed from those found in tissues in that they contained only small amounts of abnormal molecules (approximately 20%).

To determine if continued exposure of fibrils to N-proteinase could modify fibril structure, hieroglyphic fibrils were formed by incubating the mixture of \( \mathrm{pCcollagen} \) and abnormal procollagen with C-proteinase and then treated with N-proteinase (12 units in 85 µl at 34 °C for 12 h). The fibrils were collected by brief centrifugation, and the pellet and supernatant fractions were examined separately. The fibrils comprised \( \alpha_1(\mathrm{I}) \), \( \alpha_2(\mathrm{I}) \), and \( \mathrm{pN}2(\mathrm{I})^{\text{ext}} \) chains and no \( \mathrm{pNa}1(\mathrm{I}) \) chains. Laser densitometry of fluorograms demonstrated that the fibrils contained 39 ± 5% S.D. (\( n = 4 \)) \( \mathrm{pNcollagen}^{\text{ext}} \) molecules in which the \( \mathrm{pNa}1(\mathrm{I}) \) chains were cleaved to \( \alpha_1(\mathrm{I}) \) chains. The supernatant of the fibrils contained \( \alpha_1(\mathrm{I}) \) (the major component), \( \mathrm{pNa}1(\mathrm{I}) \), and \( \mathrm{pN}2(\mathrm{I})^{\text{ext}} \) chains. Only small amounts of \( \alpha_2(\mathrm{I}) \) chains were detected. These results indicated that the soluble phase was a mixture of molecules containing \( \mathrm{pN}2(\mathrm{I})^{\text{ext}} \) chains and \( \mathrm{pNa}1(\mathrm{I}) \) chains that had been cleaved to \( \alpha_1(\mathrm{I}) \) chains, and molecules containing \( \mathrm{pN}2(\mathrm{I})^{\text{ext}} \) chains and intact \( \mathrm{pNa}1(\mathrm{I}) \) chains. Noteworthy, all of the \( \mathrm{pNa}1(\mathrm{I}) \) chains in fibrils were cleaved to \( \alpha_1(\mathrm{I}) \) chains by N-proteinase (Fig. 8, lane 1), and therefore, all were most likely to be located at the surface of the fibrils. Intact \( \mathrm{pNa}1(\mathrm{I}) \) chains were found in the supernatant of the fibrils (Fig. 8, lane 2). This suggested that N-proteinase may cleave the \( \mathrm{pNa}1(\mathrm{I}) \) chains more rapidly when the \( \mathrm{pNcollagen}^{\text{ext}} \) molecules are part of fibrils than when they are in free solution.

Treatment of hieroglyphic fibrils with N-proteinase (12 units in 85 µl at 34 °C for 12 h) had a dramatic effect on fibril morphology. Examination of 18 fibrils from three preparations in 10 fields of STEM view showed that the fibrils had a symmetrical transverse mass distribution (Fig. 4D) consistent with a near circular cross-section. Hieroglyphic structures were not seen. In control experiments in which the hieroglyphic fibrils were diluted with buffer not containing N-proteinase, the fibrils had a highly irregular transverse mass distribution (data not shown).

**DISCUSSION**

*In vivo*, type I procollagen is converted to type I collagen by specific enzymic removal of N- and C-propeptides by the N- and C-proteinases (Hojima et al., 1985, 1989). The collagen then spontaneously self-assembles into the cylindrical, cross-stranded fibrils characteristic of the extracellular matrix of connective tissues. In EDS type VIIIB, individuals have mutations in their type I collagen genes that result in incomplete processing of procollagen to collagen and the accumulation in tissues of \( \mathrm{pNa}2(\mathrm{I}) \) chains. Also, the tissues of these people contain collagen fibrils that are near circular in cross-section. We wanted to learn more about the pathway of events occurring between the formation of the abnormal type I procollagen, its proteolytic processing, and the way in which these abnormal molecules participate in collagen fibril formation.

We showed that a proband with EDS type VIIIB had a G to A transition in the oblige -GT- dinucleotide at the 5′ donor splice site of exon 6 in one allele of the COL1A2 gene. Consequently, half of the proband’s type I procollagen molecules contained a \( \alpha_2(\mathrm{I}) \) chain that lacked the sequences containing the N-proteinase cleavage site. N-proteinase is a conformational-dependent proteinase that will not cleave heat-denatured procollagen (Tanzawa et al., 1985). It cleaves the \( \alpha_2(\mathrm{I}) \) chains in a sequential manner such that, during the initial stages of the reaction, \( \alpha_2(\mathrm{I}) \) and \( \mathrm{pN}2(\mathrm{I}) \) chains are cleaved at about the same rate. An intermediate is formed that is slowly converted to \( \mathrm{pCcollagen} \) by cleavage of the remaining \( \alpha_2(\mathrm{I}) \) chain (Berger et al., 1985). Also, the enzyme readily cleaves a procollagen molecule constructed from three \( \alpha_2(\mathrm{I}) \) chains in which the \( \alpha_2(\mathrm{I}) \) chain is substituted by a \( \alpha_1(\mathrm{I}) \) chain (Dombrowski and Prockop, 1988). Experiments here showed that the absence of the 18 amino acids that surround and include the N-proteinase cleavage site in the \( \alpha_2(\mathrm{I}) \) chain drastically slowed the rate of cleavage of the \( \alpha_1(\mathrm{I}) \) chains. The results demonstrate that cleavage of the \( \alpha_2(\mathrm{I}) \) chains of procollagen by N-proteinase occurs at a maximal rate only when the procollagen molecule contains three \( \alpha_2(\mathrm{I}) \) chains and when the cleavage sites are in their native conformation and spatial organization.

The self-assembly of collagen into fibrils is similar to other protein self-assembly systems, such as tobacco mosaic virus (Lauffer, 1975), actin filaments (Frieden and Goddette, 1983; Pollard and Cooper, 1986; Frieden, 1989), and microtubules (Tamasheff and Grisham, 1980; Mitchison and Kirschner, 1984; Olmsted, 1986) in that it is a spontaneous, entropy-driven process in which the driving force is the displacement of solvent molecules bound to the monomers of protein (Lauffer, 1975). Type I collagen fibrils are cylindrical, needle-like (Kadler et al., 1990a; Birk et al., 1989) and are near circular in cross-section (Kadler et al., 1990b; Birk et al., 1989). Type I \( \mathrm{pCcollagen} \) self-assembles in vitro into extended sheet-like structures of thickness 8 nm in which the N-propeptides are found at the surface of the sheets in a “folded-back” conformation (Holmes et al., 1991). The hieroglyphic structures formed here by copolymerization of collagen and \( \mathrm{pNcollagen}^{\text{ext}} \) had a morphology that was intermediate between that of cylinders and sheets. As reviewed by Oosawa and Asakura (1975), molecules that polymerize to form similar structures can copolymerize if they have similar polymer-forming tendencies. The fact that a single population of fibrils was seen, as opposed to distinct populations of sheets and fibrils, demonstrates that the hieroglyphs were copolymers of...
collagen and pNcollagen®. Treatment of the hieroglyphic fibrils with N-proteinase resulted in cleavage of all the pNα1(I) chains in pNcollagen® molecules demonstrating that the N-propeptides were located exclusively at the fibril surface. We conclude that the hieroglyphic nature of the fibrils was the result of incorporation of large amounts of pNcollagen® and close packing of the N-propeptides at the fibril surface.

Treatment of the hieroglyphic fibrils with N-proteinase resulted in cleavage of the pNα1(I) chains in pNcollagen® molecules and had drastic consequences on fibril morphology. The remarkable observation that hieroglyphic fibrils were resolved to cylinders lends support to the suggestion that there is considerable fluidity between collagen molecules within a collagen fibril (Chapman, 1989). That the relative proportions of collagen and pNcollagen® changed little after cleavage of the pNα1(I) chains by N-proteinase, and that the partially cleaved pNcollagen® molecules were present in the large diameter (small surface area/volume ratio) fibrils, suggested that some of the abnormal molecules must have been located within the body of the cylindrical fibril. The ability of the partially cleaved N-propeptides to be incorporated into the fibril suggests that they undergo a conformational change following cleavage of the pNα1(I) chains. The nature of this conformational change is unknown but one possibility is that the N-propeptides, instead of being folded back, straighten following cleavage of the pNα1(I) chains by N-proteinase.

Collagen molecules are divided into 4.4 D units and associate in parallel array so that, in the fibril, they are staggered by one or more D units relative to their nearest neighbor. This arrangement of collagen molecules generates regions of gap and overlap in the fibril. The “folded” conformation of the N-propeptides of pNcollagen molecules presumably prevents this side-by-side association whereas an “extended” conformation would not.

Wirtz et al. (1990) showed that the pNα1(I) chains in the abnormal pNcollagen from an individual with EDS type VIIIB were cleaved in vivo in the region of the α1(I) N-proteinase cleavage site and that the cleaved α1(I) N-propeptides remained noncovalently associated with the pNα2(I) chain. It was not determined whether the pNα1(I) chains were cleaved by N-proteinase or by another protease. We showed here that N-proteinase in elevated concentrations can cleave the pNα1(I) chains in the abnormal molecules and that these partially processed molecules copolymerize with collagen to form roughly cylindrical fibrils. However, even at the highest concentrations of N-proteinase that were experimentally practical, cleavage of all the pNα1(I) chains to α1(I) chains in pNcollagen® molecules in solution could not be attained. Yet, treatment of hieroglyphic fibrils with N-proteinase did result in cleavage of all the pNα1(I) chains that were in fibrils. Although not conclusive, these observations suggest that the rate of cleavage of the pNα1(I) chains by N-proteinase is greater when the N-propeptides are at the surface of a collagen fibril.

Two scenarios are possible for the assembly of collagen fibrils with near circular cross-sections in tissues of people with EDS type VIIIB. In the first, cleavage of the abnormal molecules by N-proteinase occurs prior to fibril formation. The partially cleaved pNcollagen® molecules have a low affinity for the fibril, but once bound they are buried in the body of the fibril. Fibrils formed in this way have a small fraction of pNcollagen®. In the second, collagen and pNcollagen® copolymerize, and the N-propeptides are restricted to the surface of the fibril. The intact pNcollagen® has a high affinity for the fibril so that the fibrils formed are approximately 1:1 copolymers of collagen and pNcollagen. The exposed α1(I) N-propeptides are then readily cleaved by N-proteinase and the partially cleaved pNcollagen® molecules are incorporated into the body of the fibril. Whereas both sequences of events lead to the formation of fibrils with near circular cross-sections, the copolymerization of collagen and pNcollagen® and subsequent cleavage by N-proteinase provides an explanation for the large amounts of pNα2(I) chains in the tissues of individuals with EDS type VIIIB.

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