

# A Patient with Ehlers-Danlos Syndrome Type VI Is a Compound Heterozygote for Mutations in the Lysyl Hydroxylase Gene

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## Abstract

In the present study, we have isolated and sequenced the complementary DNAs of two mutant alleles for lysyl hydroxylase (LH) in fibroblasts from one patient (AT750) with Ehlers-Danlos syndrome type VI (EDS VI). We have identified a putative mutation in each allele which may be responsible for the patient's decreased LH (normalized to prolyl hydroxylase) activity (24% of normal). Intermediate levels of LH activity were measured in the patient's parents, who are clinically normal (father 52%; mother 86%). After the cloning of cDNAs and amplification by PCR, sequence analysis revealed two equally distributed populations of cDNAs for LH in the AT750 cell line. Each allele revealed different but significant changes from the normal sequence. In one allele (allele 1), the most striking change was a triple base deletion that would result in the loss of residue Glu<sub>532</sub>. The most significant difference in the other allele (allele 2) was a G → A change which would produce a Gly<sub>678</sub> → Arg codon change in a highly conserved region of the enzyme. Restriction analysis identified that allele 1 was inherited from the proband's mother and allele 2 from the father. This study represents the first example of compound heterozygosity for the LH gene in an EDS VI patient, and it appears that there is an additive effect of each mutant allele on clinical expression in this patient. (*J. Clin. Invest.* 1994; 93:1716–1721.) Key words: collagen disease • compound heterozygote • linkage • molecular sequence data • phenotype

## Introduction

Lysyl hydroxylase (LH)<sup>1</sup> is a procollagen post-translational modifying enzyme that hydroxylates lysine residues at the Y position of Gly-X-Y peptide sequences (1). Hydroxylysine residues provide sites for intermolecular covalent crosslinks that are critical for the tensile strength of collagen fibrils (2). The hydroxylated lysine residues are also sites of attachment for galactosyl and glucogalactosyl side chains; the functions of these carbohydrate modifications are unclear although they may be important in the regulation of crosslink formation and

fibrillogenesis (3). LH exists as a homodimer ( $\alpha_2$ ) of 85,000-mol wt subunits, and requires  $\alpha$ -ketoglutarate, Fe<sup>2+</sup>, ascorbate, and O<sub>2</sub> as cofactors (1, 4, 5). The importance of this enzyme is demonstrated in patients diagnosed with Ehlers-Danlos syndrome type VI (EDS VI) (6–8). Owing to a deficiency of LH activity, these patients exhibit signs of collagen pathology that include neonatal onset of kyphoscoliosis, joint laxity, and muscle hypotonia. Other manifestations consist of skin hyperextensibility, easy bruisability, and ocular fragility (9, 10). The potential lethality of the disease is a reported tendency for spontaneous arterial rupture (10). Clinical diagnosis of EDS VI is confirmed by an assay of LH activity in cultured skin fibroblasts (11).

The genomic sequence of LH has not yet been determined, but the sequence of a full-length cDNA for LH from human placenta was recently reported (12). We have also sequenced a full-length cDNA for LH from a normal cultured dermal fibroblast cell line GM05659 (13). These sequences, apart from apparent polymorphisms, appear to have identical coding regions and provide a basis for mutational analysis in the LH cDNA from patients with EDS VI. Clinical and biochemical evidence suggest heterogeneity at the LH locus in producing EDS VI (14–16). Recent studies confirm this at the molecular level with two reported cases of different homozygous recessive mutations in LH cDNA, each in two siblings with EDS VI; these include a premature termination codon (17) and an 800-bp duplication (18), which may be responsible for the disease. Analysis of the location of the different types of mutations responsible for LH deficiency in the LH cDNA will also help to characterize the functional domains of the enzyme's primary structure. In the present study, we report the cloning and sequencing of full-length cDNAs (3 kb) for LH isolated from cultured dermal fibroblasts from a third patient with EDS VI (AT750). We have identified two novel mutant alleles for LH, which indicates that the patient is a compound heterozygote for EDS VI. The parental origin of each mutation correlates with partially reduced LH activity in their fibroblast strains and has confirmed the heterozygosity of the mutation. This represents the first reported example of compound heterozygosity in the LH gene, and it appears that a significant mutation in each allele contributes to this patient's disease phenotype.

## Patient summary

Detailed clinical information on the proband (AT750), up to the age of 6 yr, has been reported (19, 20). The patient is a male of Mexican-American ancestry born full term on 28 January 1978, without premature rupture of fetal membranes, to a non-consanguineous couple. He was noted at birth to exhibit hemifacial asymmetry and dislocation of the right wrist and hip. The patient exhibited other characteristic features of EDS VI including myopia, hyperextensible, soft and velvety skin, and hypotrophic wound healing. The patient's surgical history in-

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Received for publication 9 June 1993 and in revised form 31 December 1993.

1. Abbreviations used in this paper: EDS VI, Ehlers-Danlos syndrome type VI; LH, lysyl hydroxylase; nt, nucleotide(s); PH, prolyl hydroxylase; RT, reverse transcriptase

*J. Clin. Invest.*

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0021-9738/94/04/1716/06 \$2.00

Volume 93, April 1994, 1716–1721

cludes bilateral herniorrhaphy and orchidopexy at 6 mo of age and Harrington rod placement for kyphoscoliosis at 3 yr of age. He was demonstrated to be ascorbate-responsive and did well from the age of 6 to ~ 14 yr when he stopped taking ascorbate (15, 16, 19, 20). At 15 yr of age he had spontaneous arterial rupture into his upper thigh. Bilateral venous thrombosis resulted from compression stasis requiring fasciotomies. Pharmacological amounts of ascorbate (5.0 g/d) were reinstituted and wound healing progressed normally. He is currently 16 yr old and doing well in the 10th grade of high school.

The diagnosis of EDS VI was supported by amino acid analysis of his skin which showed zero hydroxylysine per 1,000 residues with normal values being 5/1,000. The diagnosis of EDS VI was verified by assay of LH activity from cultured dermal fibroblasts which showed that the patient had a LH/prolyl hydroxylase (PH) ratio that was 24% of the control values. PH was used as a reference since the activity of this enzyme is shown to be normal in EDS VI patients (15, 16, 19). An autosomal recessive mode of inheritance was deduced from the same assays performed on his parents' dermal fibroblasts which showed 52% and 86% LH, relative to PH activity, in the father and mother, respectively. Neither parent had clinical evidence for EDS VI.

## Methods

**Tissue culture.** Human dermal fibroblasts from a normal donor (GM05659, Institute for Medical Research, Camden, NJ), an EDS VI patient (AT750), his mother (ST1129), and his father (PT1130) were grown to confluency in DME (Gibco-BRL, Gaithersburg, MD) supplemented with 20% calf serum (Gibco-BRL).

**cDNA synthesis.** Dermal fibroblasts were trypsinized from tissue culture plates and harvested. By using  $\sim 1 \times 10^6$  cells per prep, poly(A) mRNAs were isolated using Micro-Fast Track Kit (Invitrogen, San Diego, CA) according to the manufacturer's recommendation. cDNAs were synthesized with a cDNA Cycle Kit (Invitrogen) by suggested protocol with the exception that avian myeloblastosis reverse transcriptase (RT; Seikagaku America, Rockville, MD) was used instead of the RT supplied by the kit. This required that the reaction be carried out at 37°C instead of 42°C.

**PCR.** The following 18- or 20-oligomer PCR primers were constructed according to the cDNA sequence for human dermal fibroblast LH with attached Eco RI restriction sites (given in *italics*):

- 1(A) *GCGAATTC*\*ATACCTCGGCCATGCGGC(\*nt14);
- 1(B) *GCGAATTC*\*CACCAGTTTGCTGAGGTT(\*nt528);
- 2(A) *GCGAATTC*\*AACCTCAGCAAAGTGGTG(\*nt511);
- 2(B) *GCGAATTC*\*TTCACAGACTGGTACTCGCT(\*nt1067);
- 3(A) *GCGAATTC*\*AGCGAGTACCAGTCTGTGAA(\*nt1048);
- 5(B) *GCGAATTC*\*CCACAGGCAAGTAGAAGA(\*nt2823).

Primers A are based on the sense strand and primers B are based on the complementary strand. Nucleotide (nt) numbering is based on the cDNA sequence of LH from dermal fibroblasts (13), GenBank accession number M98252. PCR reactions were carried out in 50- $\mu$ l volumes using either 0.10  $\mu$ M of each primer and 0.25 mM each of dNTPs (dATP, dCTP, dGTP, dTTP) or 0.33  $\mu$ M primer and 0.5 mM each of dNTPs with 2.5 U of Taq polymerase (Gibco-BRL) as previously described (21). The reaction cycles were 94°C for 1 min 10 s, 52°C for 2 min 10 s, and 72°C for 6 min with 10-s extensions per cycle for 40 cycles. Each set of PCR reactions included appropriate control reactions of zero template to monitor for possible PCR contaminations by foreign DNA.

**cDNA cloning.** PCR products of different A and B primer combinations were visualized on a 1% agarose-ethidium bromide gel. DNAs of the expected sizes were excised and purified using Magic PCR Preps (Promega Corp., Madison, WI) columns. To create ligatable overhanging ends, the cDNAs, which contained no internal Eco RI sites, were restricted with Eco RI (Promega Corp.) for 3 h, heat-denatured at 70°C for 5 min, and purified using Magic DNA Clean-Up (Promega Corp.) purification columns. M13mp18 bacteriophage (Eastman Laboratory Research Products, New Haven, CT) was restricted with the same enzyme for 1 h, heat-denatured, phosphatased with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 30 min, heat-denatured, and gel purified. cDNAs and M13mp18 in the ratio of ~ 2:1 were ligated using T4 DNA Ligase (Promega Corp.) overnight at 16°C. Ligated products were transformed into *Escherichia coli* strain TG1 following the method described by Hanahan (22) and incubated overnight on H-top plates. Positive colonies were picked by blue-white selection (23) with further verification by single-stranded sequencing. The number of selected cDNA clones designated as follows, by size and PCR primers, includes three full-length clones of 2809 bp (1A-5B) and 10 partial cDNA clones including seven clones of 2,312 bp (2A-5B) and one clone each of 1,775 bp (3A-5B), 1,053 bp (1A-2B), and 514 bp (1A-1B).

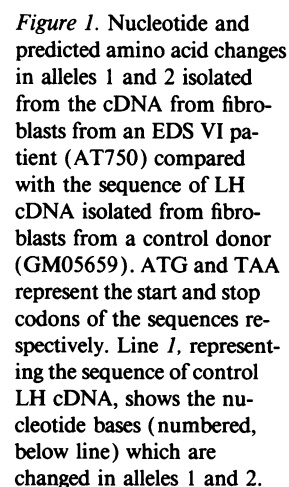
**cDNA subcloning.** Owing to the tendency of M13 phages with inserts larger than ~ 200 nt to give rise to progeny with large-scale deletions (24), the three full-length clones were subcloned into a pFlag (Eastman Laboratory Research Products) multipurpose expression vector. This was performed as previously described using Eco RI to create "sticky ends" in both cDNA insert and pFlag before ligation. Double-stranded cDNAs for the subcloning were generated from plasmid minipreps (Promega Corp.) of the three full-length M13 clones. *E. coli* strain TG1 was again used for the transformation, but because no blue-white selection exists for positive pFlag clones, random colonies were picked from overnight culture plates and grown up for isolation by plasmid minipreps. The plasmids were restricted with Eco RI and the positive clones with the correct size inserts (2809 bp) were identified on a 1% agarose-ethidium bromide gel.

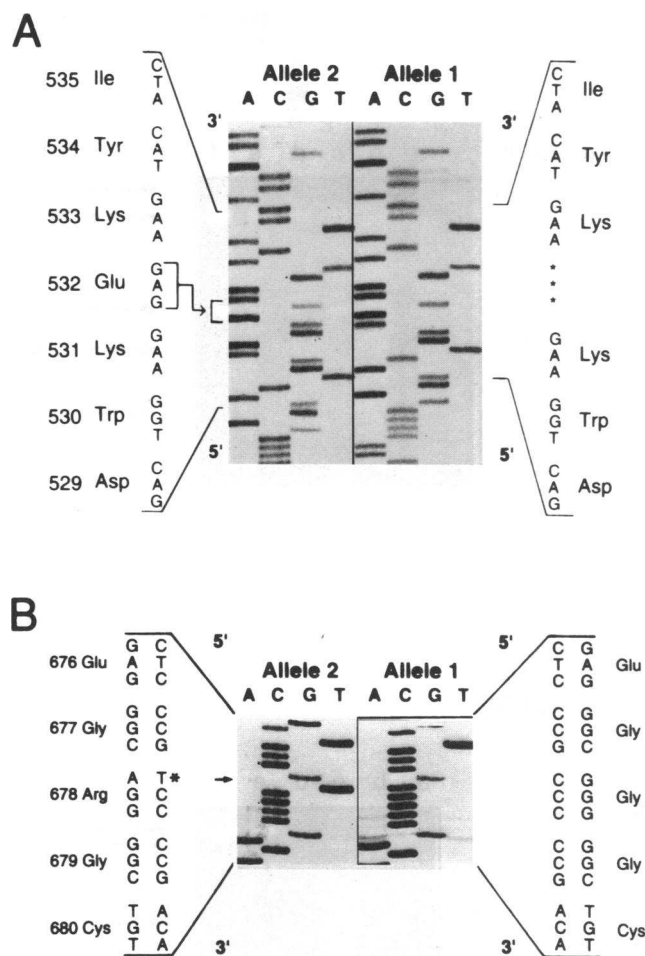
**DNA sequencing.** Single-stranded phagemids for sequencing were prepared from M13 clones by the standard procedure (23). Single-stranded phagemids of the pFlag clones were made with the use of helper phage (Eastman Laboratory Research Products) following the supplier's instructions. Sequencing was performed using Sequenase Version 2.0 kit (United States Biochemicals, Cleveland, OH) according to the dideoxy method described by Sanger et al. (25). The clones were sequenced using a series of synthetic 18-mer primers based on normal LH cDNA sequence (GenBank M98252) and were of the correct strandedness depending on clone orientation. The primers beginning at nt 14, 129, 329, 472, 653, 776, 1048, 1261, 1499, 1734, and 2006 were based on the sense strand, and for the antisense strand, sequences were read from primers at nt 2259, 2023, 1821, 1517, 1280, 1067, 827, 669, 490, 404, and 146.

**Restriction digests.** First-strand cDNA was synthesized from fibroblasts from a normal donor (GM05659), proband (AT750), mother (ST1129), and father (PT1130). Amplification of the 704-bp region covering both the triplet deletion (nt 1618–1620) observed in allele 1 and the G<sub>2056</sub> → A change observed in allele 2 was carried out using as 5' primer, the sequence between nt 1498 and 1515, and as 3' primer, the sequence complementary to nt 2185–2202. A similar region was amplified in the individual cDNA clones of alleles 1 and 2. PCR was performed as described and the gel-purified 704-bp fragments were digested at 37° with either Mbo II (1 h) or Alw N1 (16 h). At digestion times > 30 min, Mbo II produced an unpredicted fragment at ~ 300 bp, suggesting that this enzyme was cutting less specifically at a second recognition sequence. This was accompanied by a corresponding decrease in the 507-bp fragment. In contrast, Alw N1 required an overnight digestion for complete restriction. The digests were electrophoresed on a 3% NuSieve-1% agarose gel and visualized by ethidium bromide-UV fluorescence.

**Sequence analysis of allele 1.** The control LH cDNA sequence codes for a 727-amino acid protein beginning with an 18-amino acid signal peptide. It contains 10 cysteine residues, some of which are thought to be important for Fe<sup>2+</sup> binding (12). It also contains 4 possible sites for asparagine-linked oligosaccharide attachment (12). These cysteine and asparagine residues were unchanged in allele 1. The most significant change in allele 1 (Fig. 1, *boxed*) from control LH cDNA is a triplet deletion of bases GAG at nt 1618–1620 that would result in the in-frame loss of residue Glu<sub>532</sub> (Fig. 2). The predicted amino acid loss is positioned 6 amino acids 5' to one of the four asparagine-linked oligosaccharide attachment sites.

As shown in Fig. 3 *B(i)*, Mbo II digests the cDNA from the control nucleotide sequence (lane *a*) into two fragments of 507 and 197 bp. In allele 1, the 197-bp fragment is foreshortened to 194 bp because of the deleted triplet and introduces a new Mbo II site which produces smaller fragments of 128 and 66 bp (lane *b*). Allele 2 has a similar Mbo II restriction pattern (lane *c*) as the control allele. The restriction fragments of 507, 197, 128, and 66 bp observed in the cDNA from the proband (lane *d*) confirmed that the proband was a heterozygote for the normal

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**Figure 2.** (A) Nucleotide sequence of the region of cDNA in which the triplet GAG coding for Glu<sub>532</sub> is deleted in allele 1 but present in allele 2 and control LH cDNA sequence. The asterisks represent deleted bases in allele 1 corresponding to codon GAG ( $\rightarrow$  [ ]) in allele 2. Note: direct repeats (underlined as follows) flanking and including deleted triplet **\*\*\***(*gag*) in allele 1 5'GAAGgagAAG3'. (B) Nucleotide sequence of the region of cDNA in which a single base mutation G  $\rightarrow$  A in allele 2 results in a Gly<sub>678</sub>  $\rightarrow$  Arg change. The sequence shown is the noncoding strand. The asterisk represents, in the coding strand, the substitution of an A in allele 2 for the normal G present in allele 1 and control LH cDNA sequence which converts the codon of GGG for glycine at amino acid residue 678 to AGG, a codon for arginine. Note: this occurs in a CG dinucleotide region resulting in CG  $\rightarrow$  CA\*.

and GAG-deleted nucleotide sequence. A pattern similar to the proband was observed with the cDNA from the proband's mother (lane *f*), whereas the father's cDNA gave a normal restriction pattern (lane *e*). In Fig. 3 B(ii), Alw N1 does not cut the 704-bp cDNA fragment from the control allele (lane *a*) or allele 1 (lane *b*), whereas it digests allele 2 into two fragments of 562 and 142 bp (lane *c*). Digestion of the proband's cDNA gave three fragments of 704, 562, and 142 bp (lane *d*), confirming that the proband was a heterozygote for the new Alw N1 restriction site. A similar restriction pattern of the father's cDNA showed the presence of allele 2 (lane *e*) which was absent in the mother (lane *f*). These digests confirm that both abnormal alleles are present in the proband's cDNA and that

the proband has inherited allele 1 (E532del) from his mother and allele 2 (G678R) from his father.

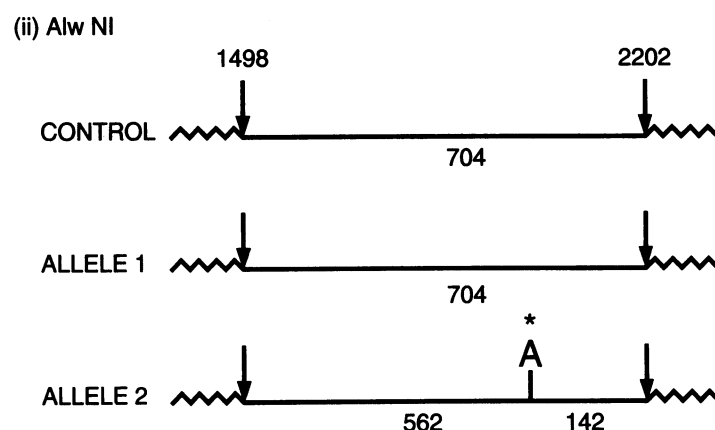
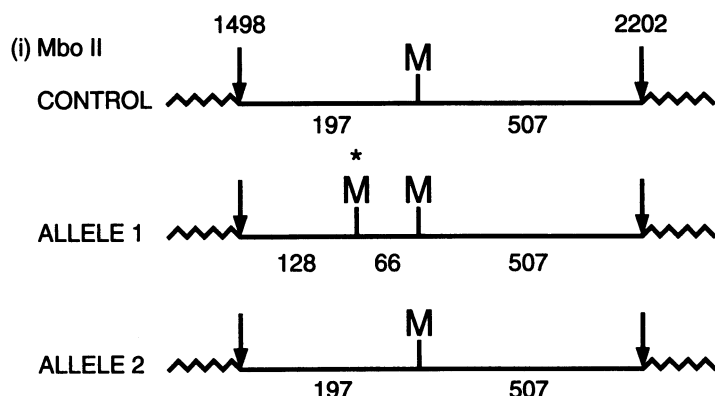
## Discussion

The biochemical basis for EDS VI was first described in 1972 by Pinnell et al. (6) in which they demonstrated that dermal fibroblasts from patients with EDS VI had decreased LH activity (6). In the present study, we describe one patient with the clinical features of EDS VI whose cultured skin fibroblasts have only 24% of LH to PH activity compared with that of control (19). Through RT-PCR techniques, we have cloned the cDNAs from the two alleles of LH from this patient and have identified different mutations in each allele that may contribute additively to the patient's clinical phenotype.

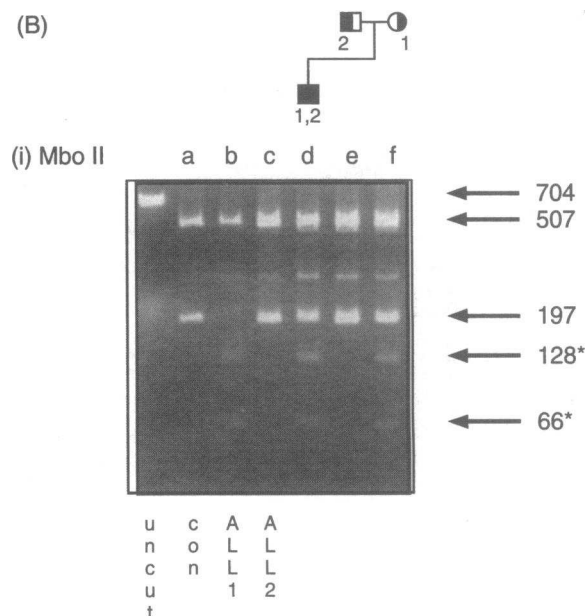
We have shown that one allele (allele 1) isolated from this patient's LH cDNA contains a significant triple-nucleotide deletion that would result in the predicted loss of a glutamic acid at residue 532 (E532del). The region in which this triple deletion occurs contains a direct symmetrical repeat of -GAAG- (Fig. 2), which may predispose this sequence, if it is uninterrupted in the LH gene, to a deletion-type mutation. This has been reported in several other such mutated sequences in which short direct repeats flank a deletion (28). In allele 1, this deletion would appear to be the major contributor to the patient's disease phenotype; however, as the tertiary structure of LH is not yet known, the precise effect of this deletion on the functional site of the enzyme cannot be presently determined. Other minor base changes identified in allele 1 include a polymorphism and a conservative base change. The significance of the Ala  $\rightarrow$  Ser codon change is presently unknown. This change has not been observed in sequence analysis of six LH alleles from control fibroblasts (13); however, it is unlikely to produce a functional change in LH since there is no charge change and this region of the protein is not conserved (27).

Sequence analysis of the other allele (allele 2) reveals both polymorphic differences from control cDNA and a significant mutation which are not present in allele 1. The most significant mutation in allele 2 is a single-base transition giving rise to a Gly<sub>678</sub>  $\rightarrow$  Arg (G678R) change. Interestingly this change of a CG<sub>2056</sub>  $\rightarrow$  CA occurs at a CpG dinucleotide site. Such CpG repeats are implicated as hot spots for mutations involving single base pair substitutions and are linked to human genetic disease (29, 30). This mutation disrupts a triple glycine peptide sequence in a highly conserved area of the primary structure of the enzyme (27). In this region between amino acids 639 and 715, the polypeptide sequences of chick and human LH are 99% identical. Significantly this highly conserved region of the LH gene containing the mutation appears to encompass the catalytic site of the enzyme (27). A recent comparison of amino acid sequences of several  $\alpha$ -ketoglutarate-coupled dioxygenases, revealed two histidine motifs that are highly conserved (27) and apparently important for catalytic activity of these enzymes. In human LH cDNA, these histidine residues are at positions 657 and 708 located within the 99% conserved carboxyl terminus. It therefore appears likely that the location of this mutation between two important histidine motifs in a highly conserved region of the carboxyl terminus contributes significantly to loss of LH activity in fibroblasts from this patient. Two other polymorphic changes in allele 2 occur at the

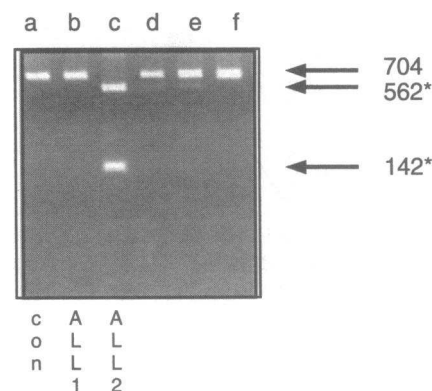
# (A) Restriction Maps



# (B)



# (ii) Alw NI



**Figure 3.** Analysis of Mbo II- and Alw NI-digested PCR products from LH cDNA of proband and his parents and the cDNA clones from the proband's two mutant alleles. (A) Restriction maps. (i) The Mbo II restriction of the PCR-amplified region in the control allele and the abnormal alleles 1 and 2. The arrows indicate the boundaries of the 704-bp sequence amplified by PCR. Fragment sizes are marked in base pairs below the line. M represents the restriction site in the control sequence and M indicates the new restriction site in allele 1. (ii) The Alw NI restriction of the similarly amplified regions represented by arrows as shown for Mbo II. Alw NI does not cut the control sequence and A indicates the new restriction site in allele 2. (B) The family pedigree is shown at the top; the mutant alleles are shaded in black and numbered as 1, and 2. (i) Mbo II digests of PCR products amplified from cDNA from a control donor (lane a), individual alleles 1 and 2 from proband (lanes b and c), proband (lane d), father (lane e), and mother (lane f) were separated by gel electrophoresis. Uncut cDNA is on the left of the gel. The sizes (bp) of the uncut and Mbo II restriction fragments are marked on the right of the gel. \*The 66- and 128-bp fragments created by the triplet deletion in allele 1. Note: at digestion times of > 30 min, Mbo II appears to cut at an unpredicted, less specific second recognition sequence producing a  $\approx$  300-bp fragment, with a corresponding decrease in the 507-bp fragment over time. (ii) Alw NI digests of PCR products amplified as described for Mbo II. \*The 142 and 562 bp fragments created by the single base change in allele 2.

third position of the triplet codon but do not change amino acid sequence.

The autosomal recessive nature of EDS VI suggested by a comparison of the LH and PH activities in the parents' fibroblasts (father 52%; mother 86%) with that of the proband (24%) was confirmed by restriction analysis showing that the E532del (allele 1) was inherited from the proband's mother and the G678R mutation (allele 2) from the father. The pres-

ence of an apparently normal allele in each parent, who are clinically normal, was also confirmed. The presence of one normal and one mutant allele would enable each parent to express normal, heterozygous, and homozygous abnormal LH dimers in contrast to the proband, whose array of holoenzymes (E532del/E532del; E532del/G678R; G678R/G678R) would all be abnormal. The partially reduced levels of LH activities in the parental cells (19) have been recently confirmed in our

laboratory (S. Murad, unpublished observations); a comparison of the LH activities in each parent's fibroblasts with their respective mutations may reflect the degree of the disruption caused by each mutation on the enzyme's functional sites. Our findings suggest that the paternal G678R mutation in the conserved region of LH is more deleterious to enzyme function than the E532del in the maternal allele; however, the structure-function mechanism remains to be determined.

In conclusion, we have found two different mutations for the LH gene in fibroblasts from a patient with EDS VI. This study represents the first example of compound heterozygosity for the LH gene identified in a patient with EDS VI and it would appear that there is an additive effect of each mutant allele on clinical expression in the patient. Determination of the catalytic activity of these alleles, expressed individually and together in a system such as baculovirus will enable us to assess the relative effect of each mutation and thus further our understanding of the structural biology of this important enzyme in collagen biosynthesis.

## Acknowledgments

The authors would like to thank Linda Walker for isolation of the cDNAs and Susan Combs for her help with tissue culture in this study. They would like also to thank Dr. Guy Salveson, Long Quan, and Scott Snipas in the Department of Molecular Pathology at Duke University for their help throughout the project. They also acknowledge the help of Dr. Richard Sontheimer in obtaining the skin biopsies from the parents of the proband. In addition, the authors would like to thank Dr. Charlotte Phillips and Dr. Richard Wenstrup for critical reading of the manuscript for this article. They gratefully acknowledge the help of Genia Holder and Margaret Poole in typing the manuscript.

This work was supported in part by research grant AG-10215 from the National Institute on Aging (Dr. Yeowell), Basic Research grant 1-Fy93-0362 from the March of Dimes Birth Defects Foundation (Dr. Yeowell), research grant AR17128 from the National Institute of Arthritis, Musculoskeletal and Skin Diseases; and a Loo Stead Research Scholarship (Dr. Ha). Dr. Yeowell is a Senior Fellow of the Center for the Study of Aging and Human Development at Duke University Medical Center.

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