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Deletions within COL7A1 Exons Distant from Consensus Splice Sites Alter Splicing and Produce Shortened Polypeptides in Dominant Dystrophic Epidermolysis Bullosa

Anavaj Sakuntabhai,[1] Nadja Hammami-Hauasli,[3] Christine Bodemer,[4] Ariane Rochat,[5] Catherine Prost,[6] Yann Barrandon,[5] Yves de Prost,[4] Mark Lathrop,[1] Fenella Wojnarowska,[2] Leena Bruckner-Tuderman,[3] and Alain Hovnanian[1]

[1]The Wellcome Trust Centre for Human Genetics, University of Oxford, and [2]Department of Dermatology, Oxford Radcliffe Hospital, Oxford; [3]Department of Dermatology, University of Münster, Münster; and [4]Department of Dermatology, Necker Hospital, [5]Laboratory of Cellular Differentiation, École Normale Supérieure, and [6]Department of Histology, Saint-Louis Hospital, Paris

Summary

We describe two familial cases of dominant dystrophic epidermolysis bullosa (DDEB) that are heterozygous for deletions in COL7A1 that alter splicing, despite intact consensus splice-site sequences. One patient shows a 28-bp genomic deletion (6081del28) in exon 73 associated with the activation of a cryptic donor splice site within this exon; the combination of both defects restores the phase and replaces the last 11 Gly-X-Y repeats of exon 73 by a noncollagenous sequence, Glu-Ser-Leu. The second patient demonstrates a 27-bp deletion in exon 87 (6847del27), causing in-frame skipping of this exon; consensus splice sites, putative branch sites, and introns flanking exons 73 and 87 showed a normal sequence. Keratinocytes from the probands synthesized normal and shortened type VII collagen polypeptides and showed intracellular accumulation of type VII procollagen molecules. This first report of genomic deletions in COL7A1 in DDEB suggests a role for exonic sequences in the control of splicing of COL7A1 pre-mRNA and provides evidence that shortened type VII collagen polypeptides can alter, in a dominant manner, anchoring-fibril formation and can cause DDEB of differing severity.

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Address for correspondence and reprints: Dr. Alain Hovnanian, The Wellcome Trust Centre for Human Genetics, Windmill Road, Headington, Oxford OX3 7BN, United Kingdom. E-mail: alain.hovnanian@well.ox.ac.uk

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Introduction

Epidermolysis bullosa (EB) comprises a heterogeneous group of hereditary skin diseases characterized by blistering of the skin and mucous membranes after mild trauma (Fine et al. 1991; Bruckner-Tuderman 1993). The dystrophic forms of EB (DEB) demonstrate cleavage beneath the lamina densa, at the level of anchoring fibrils (AFs), which show various abnormalities (Tidman and Eady 1985; McGrath et al. 1993). DEB occurs in both a dominantly inherited form (DDEB) and a recessively inherited form (RDEB), each form being subdivided into different clinical subtypes. Close linkage has been established between the type VII collagen gene (COL7A1) on chromosomal region 3p21, which encodes the constitutive molecules of AFs (Burgeson et al. 1990), and both DDEB and RDEB (Ryynänen et al. 1991; Al-Imara et al. 1992; Hovnanian et al. 1992; Dunnill et al. 1994; Naeyaert et al. 1995). Distinct COL7A1 mutations have been identified in DDEB and RDEB, which allow for some genotype-phenotype correlations to emerge (reviewed in Järvikallio et al. 1997).

DDEB comprises two major forms, the Cockayne-Touraine variant and the Pasini variant, which display generalized blistering, predominantly at sites of trauma. The other subtypes of DDEB include the pretibial form, which is a rare and localized clinical variant; the pruriginosa type, which is associated with severe pruritus, prurigo nodules, and scarring of the extensors of the extremities; and Bart syndrome, which presents with congenital absence of skin on the lower extremities (Fine et al. 1991). Blistering of the skin leads to scarring and milia, sometimes associated with albopapuloid lesions; nails are frequently dystrophic; and oral blistering can occur. Electron-microscopic studies show AF abnormalities, which can be reduced in number and rudimentary in appearance. Immunofluorescence staining using LH7:2 antibody against type VII collagen is sometimes reduced but is often normal (McGrath et al. 1993).

Dominant transient bullous dermolysis of the newborn is a particular form of the disease, with neonatal blistering that heals within the first months or 1st year of life and is associated with transient intracellular retention of type VII collagen (Fine et al. 1991). COL7A1 mutations have been identified in all known DDEB forms, indicating that DDEB subtypes are allelic variants caused by different mutations in the same gene (Christiano et al. 1994*c,* 1995*a,* 1995*b,* 1996*a,* 1996*b,* 1996*c;* Kon et al. 1997*a*, 1997*b;* Lee et al. 1997). Apart from a splice-site mutation identified in a family with dominantly inherited transient bullous dermolysis of the newborn (Christiano et al. 1997*b*), COL7A1 mutations described so far in DDEB all involve glycine substitutions within the triple-helical domain of the molecule. By analogy with glycine substitutions identified in other collagen genes in inherited connective-tissue disorders reviewed by Byers (1990) and Kuivaniemi et al. (1991), substitutions of these glycine residues in COL7A1 are thought to have a dominant-negative effect on both type VII collagen assembly and AF formation. In contrast, a majority of mutations in RDEB result in premature-termination codons (PTCs), leading to mRNA instability and absence of type VII collagen protein synthesis (Hilal et al. 1993; Christiano et al. 1994*a,* 1995*c,* 1997*a;* Hovnanian et al. 1994, 1997; Dunnill et al. 1996; Hammami-Hauasli et al. 1997). However, glycine substitutions in the collagenous domain of type VII collagen also have been reported in RDEB, indicating that some glycine substitutions act in a recessive manner (Christiano et al. 1996*c;* Hovnanian et al. 1997; Kon et al. 1997*a*).

Collagen VII molecules are homotrimers made of proα1(VII) chains. Each chain consists of a large (145 kD) central triple-helical collagenous domain flanked by an amino-terminal (145 kD) and a carboxy-terminal (30 kD) noncollagenous domain (Burgeson 1993). The central domain contains a repeating Gly-X-Y collagenous sequence, which folds into triple-helical conformation. The Gly-X-Y motif is interrupted 19 times by noncollagenous regions that are thought to provide flexibility to the molecule. AFs are formed by antiparallel association of homotrimers, through their NC-2 domains and by lateral aggregation of the collagenous domains of the molecules. They provide critical cohesion of the epidermis to the dermis, via interactions both with components of the lamina densa and with anchoring plaques in the dermis (Burgeson et al. 1990).

A growing body of evidence indicates that exons contain sequences that assist in exon definition during splicing in vertebrate genes (Reed and Maniatis 1986). The role of such sequences in splice-site selection was described initially in genes undergoing differential splicing and now has been documented in several human disease genes (Cooper and Mattox 1997). These observations have supported the "exon definition" model whereby splice-site recognition requires pairing between 5′ and 3′ splice sites across the exon, via binding of small nuclear ribonucleoproteins and associated specific transacting factors that form the spliceosome (Berget 1995). Among the exonic sequences involved in splice-site selection are "exonic splicing enhancers" (ESEs), which often are purine-rich sequences that bind serine and arginine-rich (SR) proteins during spliceosome assembly. ESE sequences also include AC-rich enhancers (ACEs) and other sequences that do not resemble purine-rich ESEs or ACEs (Coulter et al. 1997). In support of the role of exons in splicing, point mutations in ESEs sequences that cause skipping of the exon harboring the mutation recently have been reported in human disease genes (reviewed in Cooper and Mattox 1997). Large deletions or insertions within exons also have been shown to affect splicing and to cause exon skipping, suggesting that the size and/or secondary structure of the exon also are critical for exon recognition (Reed and Maniatis 1986; Matsuo et al. 1992).

Here we have studied two familial cases of DDEB in which the disease severity showed marked improvement with age, and we have identified genomic deletions in exons 73 and 87 of COL7A1. Although these defects are located some distance from the consensus splice sites, splicing of the corresponding exon is altered and causes in-frame skipping of collagenous sequences. As a consequence, shortened proα1(VII) chains are produced, and keratinocytes in culture show intracellular accumulation of type VII collagen polypeptide. These data reveal that the integrity of exons 73 and 87 is essential for normal splicing of COL7A1 pre-mRNA and indicate that shortened proα1(VII) chains can impair, in a dominant manner, AF formation.

Subjects and Methods

Clinical Features of Subjects

Family 1.—The proband is a 32-year-old White South African woman. She is the third child of unrelated and unaffected parents. She was noted at birth to have hemorrhagic blisters underneath both thumbnails and to have dystrophic nails. During childhood, she had trauma-induced blisters on her knees, elbows, ankles, and the dorsum of her feet. She also presented with hemorrhagic blisters in her mouth. Blistering was at its most severe during early childhood and diminished markedly at the onset of adulthood. On examination at age of 32 years, she had minimal milia and scarring but had markedly dystrophic fingernails and toenails (fig. 1*a*). The patient recently gave birth to her first child, an affected daughter who displayed congenital absence of skin of the right leg and foot (fig. 1*b*). The skin condition of her daughter improved after birth, but blistering still

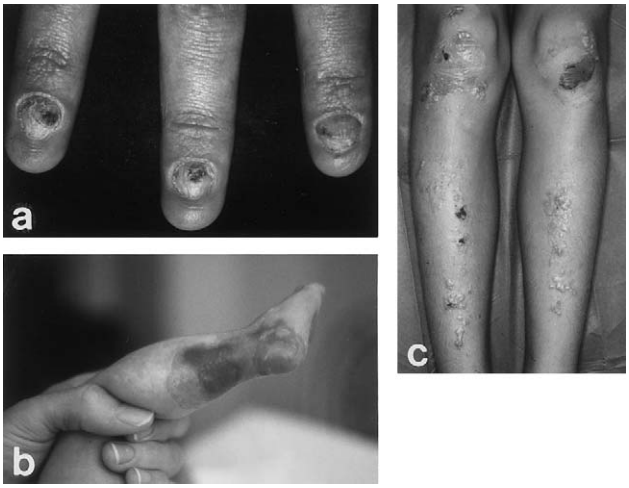

Figure 1 Clinical presentation of patients and of patient 1's daughter. *a*, Patient 1 at age 32 years, showing dystrophic finger nails with minimal scarring. *b*, Patient 1's daughter, showing congenital absence of skin of the posterior aspect of the right leg and heel. *c*, Patient 2 at age 10 years, showing blistering and atrophic scarring on the pretibial area and knees, with numerous and hypertrophic milia.

occurs after relatively mild trauma. The condition of the patient and her daughter was consistent with DDEB.

Family 2.—The proband is a 10-year-old French girl who is the only child of unrelated parents. She developed trauma-induced erosions and blisters mainly on pretibial area and knees after the age of 7 mo. Some blisters and milia also developed on fingers. On examination, she had red atrophic scars on the knees, on pretibial areas, and on the ankles, with numerous and hypertrophic milia (fig. 1*c*). Pruritus was frequent. Her toenails were dystrophic; her mucous membranes were not affected. No albopapuloid lesions were observed. Her father and paternal grandmother had had dystrophic nails since childhood. They had developed blistering to a lesser extent than had the proband. In particular, blistering in her father and grandmother spared pretibial skin and had improved markedly by the onset of adulthood. Clinical examination of her father, at age of 45 years, showed nail dystrophy with neither atrophic scars nor milia. This family presented with the diagnosis of pretibial DDEB with intrafamilial variability.

PCR Amplification

After informed consent was obtained, DNA was isolated from peripheral blood leukocytes and was used as a template for amplification of genomic sequences within COL7A1. Oligonucleotide primers were designed to generate 62 PCR products spanning all 118 exons and flanking splice sites of COL7A1 (Christiano et al. 1997*c*). PCR amplification was performed by use of 100 ng genomic DNA as a template in 50 μl of reaction mixture that contained 200 μM of each dNTP, 12.5 pM of each primer, 1.5 mM magnesium chloride, 45 mM Tris pH 8.8, 11 mM ammonium sulfate, and 6.7 mM β-mercaptoethanol with 0.4 U Ampli*Taq* polymerase (Perkin Elmer). The following conditions were used: hot start, 35 cycles of 94°C for 30 s, T_m of the primers for 40 s, and 72°C for 30 s, in a MJ Research PTC-100 thermal cycler. The primer sets used to detect the mutations from genomic DNA were as follows: for exon 73, sense primer 5′-GGGTGTAGCTGTACAGCCAC-3′ and the anti-sense primer 5′-CCCTCTTCCCTCACTCTCCT-3′ (nucleotide positions 23399–23684 in the genomic sequence [Genbank accession number L23982]); for exon 87, sense primer 5′-GTCAAGGGTTGGGCTCCAGG-3′ and antisense primer 5′-TGGAAACAGGCTTGTGGGTG-3′ (nucleotide positions 25974–26377). The annealing temperatures used were 55°C and 60°C for exons 73 and 87, respectively.

The mutations were investigated by amplification of reverse-transcribed RNA from keratinocytes by use of COL7A1 cDNA primers encompassing the exons flanking the mutation.

Conformation-Sensitive Gel Electrophoresis (CSGE) Analysis

Screening for nucleotide changes in the other exons of COL7A1 and in flanking intronic sequences was performed by means of heteroduplex analysis as described by Ganguly et al. (1993). After PCR amplification, the products from the patient and a normal control subject were mixed in a 2:1 ratio. The formation of heteroduplexes was enhanced by heating the products to 98°C for 5 min, followed by heating at 68°C for 1 h. A 10-μl sample was loaded onto a gel containing 10% polyacrylamide, 15% formamide, 10% ethylene glycol, and 0.5% glycerol-tolerant buffer (USB) and was subjected to electrophoresis at 500 V for 16 h.

Keratinocyte Culture, RNA Extraction, and Reverse Transcriptase–PCR (RT-PCR)

Keratinocytes were cultured from 5-mm punch biopsies obtained from the patients, in the manner described by Rheinwald and Green (1975) and Rochat et al. (1994). Total RNA was extracted from confluent cell layers by means of the guanidium thiocyanate method described by Chomczynski and Sacchi (1987). cDNA was generated from total RNA by reverse transcription using random hexanucleotides (Pharmacia Biotech) and Moloney murine leukemia–virus reverse transcriptase (Stratagene). The following sets of exonic primers were used to amplify and sequence cDNA: sense primer 5′-GCCTGGAAGTGTGCCGAATG-3′ and antisense primer 5′-CCCCCTTAGCACCCTTGAGT-3′, to amplify exons 71–76 (nucleotide positions 5805–6352 in

the cDNA sequence [Genbank accession number L02870]) (Christiano et al. 1994*b*), and sense primer 5′-GGTCCACAGGGGTCTCCA-3′ and antisense primer 5′-CCAGGTCTCCAGCAAGGC-3′, to amplify exons 85–89 (nucleotide positions 6715–6967). The annealing temperatures used were 60°C and 55°C, to amplify exons 72–75 and exons 85–89, respectively.

Sequencing

PCR products showing abnormal electrophoretic mobility were gel purified and sequenced in the forward and reverse orientations, by means of the ABI PRISM Ampli*Taq*–reaction dye terminator cycle–sequencing kit (PE Applied Biosystems) and an Applied Biosystems model 373A automated sequencer.

Immunofluorescence (IF) Study

Indirect IF staining was performed on cryosections of punch biopsies, with the monoclonal antibody LH7:2 to the NC-1 domain of type VII collagen, diluted 1:1,000, from Sigma Immuno Chemicals (Bruckner-Tuderman et al. 1989). The FITC-labeled anti-mouse antibody (Sigma Immuno Chemicals) was used as secondary antibody. Antibodies to type IV collagen, laminin, and GB3 (BM600) and to bullous pemphigoid antiserum also were used. For IF staining of keratinocytes in vitro, cells were grown on glass coverslips and were incubated with 50 μg L-ascorbate/ml for 48 h prior to the antibody incubations (Schumann et al. 1997). The IF staining was performed with the monoclonal antibody LH-7.2 to the NC-1 domain of human type VII collagen and with polyclonal antibodies to the collagenous and the NC-2 domains (Bruckner-Tuderman et al. 1995). The FITC-labeled anti-mouse and anti-rabbit secondary antibodies were obtained from Dako.

Protein Extraction and Immunoblot Analyses

For immunoblotting, age-matched control and DDEB keratinocytes were cultured to subconfluency and then were incubated with 50 μg L-ascorbate/ml for 48 h prior to the extraction. The culture media and the cell layers were extracted separately. The medium was precipitated with ethanol in the presence of 10 mM EDTA and 1 mM Pefablock (Merck) as proteinase inhibitors (König and Bruckner-Tuderman 1992), and the cell layer was extracted with a neutral buffer containing 0.1 M NaCl, 10 mM EDTA, 20 mM Tris-HCl pH 7.4, 1% NP-40 (Sonnenberg et al. 1991). The samples were subjected to SDS-PAGE on polyacrylamide gels with a 10%–15% polyacrylamide gradient, to immunoblotting, and to a semiquantitative immunoassay using domain-specific collagen VII antibodies (König et al. 1992).

Pepsin Digestion

Fragmentation of type VII procollagen from control and DDEB keratinocytes was performed by means of limited pepsin digestion (Bruckner and Prockop 1981; Burgeson 1993). For the enzyme reaction, cell extracts were acidified by the addition of glacial acetic acid to a final concentration of 0.1 M, and the samples were incubated with 10 μg pepsin (Fluka)/ml at 5°C for 2 h. The reaction was stopped by neutralization with unbuffered Tris. After ethanol precipitation at −20°C overnight, the samples were dissolved in SDS-PAGE sample buffer and were subjected to immunoblotting.

Electron Microscopy

Skin biopsies were taken, under local anesthesia, from clinically uninvolved skin from the probands of families 1 and 2. Electron microscopy was performed on a portion of a skin biopsy fixed in 2.5% glutaraldehyde and processed by means of standard techniques as described by Eady (1985). Sections were observed under a Jeol 1200 EX transmission-electron microscope.

Results

Identification of COL7A1 Deletions

To screen for mutations within COL7A1, the 118 exons and flanking intronic sequences were amplified from genomic DNA. The PCR products were first tested on 2% agarose gels and were run on CSGE. Of the 118 exons analyzed, exon 73 and exon 87 showed abnormal migration patterns on agarose-gel tests in patients 1 and 2, respectively, suggestive of the presence of a genomic deletion in the corresponding fragments (fig. 2).

Patient 1.—PCR amplification of exon 73 showed a smaller band in addition to the band of normal size, the two bands being of comparable intensity (fig. 2*a*). Direct sequencing of the smaller band revealed a 28-bp genomic deletion in exon 73, spanning nucleotides 6081–6108 in exon 73 (6081del28) (fig. 3*a*). Sequence analysis of the band of normal size showed a normal sequence. DNA analysis of the proband's affected daughter confirmed that she had inherited the 6081del28 mutation. DNA from the patient's parents was not available for study.

Patient 2.—PCR amplification of exon 87 and flanking intron sequences showed a band of reduced size in addition to the band of normal size (fig. 2*b*). Direct sequencing of the smaller band revealed a 27-nucleotide deletion in exon 87, encompassing nucleotides 6847–6873 (6847del27) (fig. 3*b*). Direct sequencing of the band of normal size showed a normal sequence. The presence of the 6847del27 mutation at a heterozygous state was confirmed in the proband's affected father.

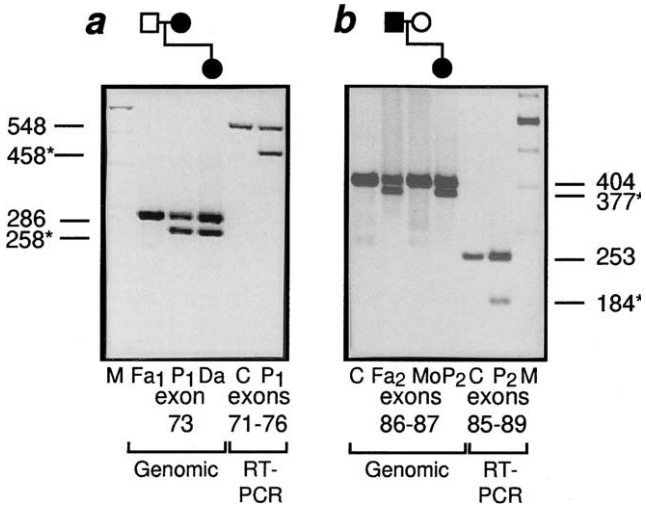

Figure 2 Genomic amplification of exons 73 and 87 from COL7A1, and RT-PCR amplification of corresponding regions in patients 1 and 2. *a,* Family 1, in which amplification of exon 73 from genomic DNA showed an extra band (258 bp) migrating faster than the band of normal size (286 bp), in both patient 1 (lanes P1) and her affected daughter (lane Da). Sequencing of this lower band showed a 28-bp deletion (6081del28) in exon 73, located 72 bp upstream of the donor splice sites of intron 73. In patient 1, RT-PCR amplification of the COL7A1 cDNA region from exons 71–76 showed a smaller band (458 bp) than would be expected from the deletion. Sequence analysis of this band confirmed 6081del28 and revealed abnormal splicing of the last 62 nucleotides of exon 73. *b,* Family 2, in which genomic amplification of exons 86 and 87 showed a smaller band (377 bp) in addition to the band of normal size (404 bp) in both patient 2 (lanes P2) and her affected father (lane Fa2). Sequencing of the lower band showed a 27-bp deletion in exon 87, located 27 bp upstream of the donor splice site of intron 87. In patient 2, RT-PCR amplification of the COL7A1 cDNA region encompassing exons 85–89 showed a band of reduced intensity and smaller size (184 bp) than would be predicted from the deletion. Sequencing of this band (184 bp) revealed skipping of exon 87. The reduction in intensity of this band may be due to variable degradation of the mutated transcript, despite the fact that the reading frame is maintained. Bands of abnormal size are denoted by an asterisk (*). Numbering of the nucleotides refers to the cDNA sequence published by Christiano et al. (1994*b*) (Genbank accession number L02870).

Abnormal Splicing of Exons 73 and 87

Since the role of internal exon sequences in splice-site selection recently has been documented in human disease, we sought to investigate splicing of exons 73 and 87 in patients 1 and 2, respectively.

Patient 1.—Amplification of exons 71–76 from reverse-transcribed total RNA from culture keratinocytes showed a smaller band in addition to the band of normal size, both bands being of comparable intensity (fig. 2*a*). Direct sequencing of the smaller band showed the deletion of nucleotides 6081–6108 and the removal of the last 62 nucleotides of exon 73 (nucleotides 6119–6180), leaving nucleotides 6109–6118 unchanged (fig. 3*a*). There was no evidence for normal splicing of exon 73 harboring the 6081del28 mutation. The abnormal splicing of exon 73 should originate from the activation of the cryptic splice donor site GT6119 within exon 73 (fig. 3*a*). The combination of the genomic deletion and the splice abnormality restores the phase and predicts the replacement of the last 11 Gly-X-Y repeats of exon 73 by noncollagenous sequence Glu-Ser-Leu (fig. 3*a*). Sequencing of the band of normal size showed a normal sequence.

Patient 2.—Amplification of exons 85–89 from reverse-transcribed RNA showed a smaller band in addition to the band of normal size (fig. 2*b*). Sequencing of the smaller band disclosed the removal of Gly2278–Gln2300 encoded by exon 87, which was consistent with skipping of this exon (fig. 3*b*). There was no evidence for normal splicing of exon 87 harboring the 6847del27 mutation.

Screening for the Mutations

The mutations that we report were not found in either 18 DDEB patients or 64 RDEB patients but were specific

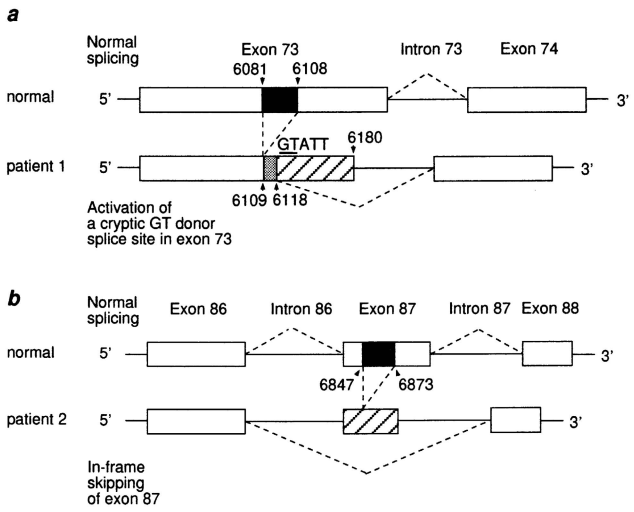

Figure 3 Schematic representation of the genomic deletions of exons 73 and 87 and associated abnormal splicing. Exons and parts of exons present in the mRNA are shown as blank boxes, genomic deletions of exons are shown as black boxes, and the exons or parts of the exons that are removed by abnormal splicing are shown as boxes with hatching. *a,* Patient 1. The 28-bp genomic deletion in exon 73 leads to abnormal splicing of the last 62 bp of this exon after the activation of a cryptic GT donor splice site (*underlined*). The 10-bp sequence (nucleotides 6109–6118) encoding the new amino acids E2028, S2029, and L2030 between the 3′ end of the genomic deletion and the cryptic GT donor splice is shown as in gray shaded. *b,* Patient 2. The 27-bp genomic deletion of exon 87 produces skipping of the entire exon 87. These splicing abnormalities occurred despite the fact that the consensus donor splice-site sequences of intron 73 and 87 remain intact.

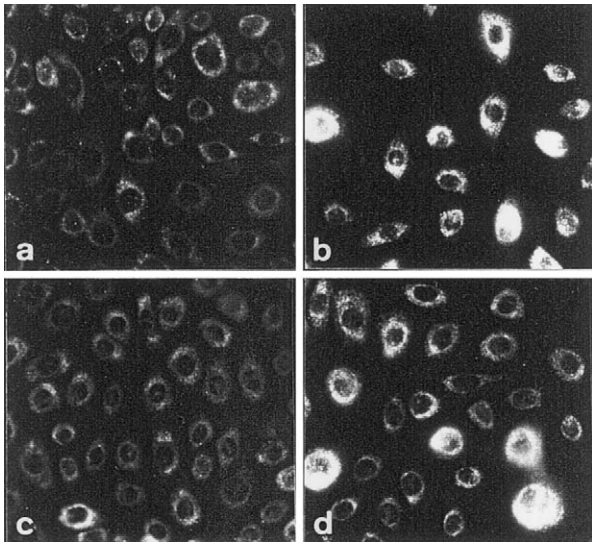

Figure 4 Indirect IF staining of control and patient's keratinocytes, with antibodies to type VII collagen. Staining of normal human keratinocytes (*a* and *c*) exhibits a weak granular intracellular IF, whereas staining of keratinocytes of patients 1 (*b*) and (*d*) clearly show increased immune reactivity, indicating intracellular accumulation of type VII procollagen.

to the two families studied here. Neither mutation was found in 50 normal controls. Complete screening of the 118 exons of COL7A1 and of their flanking splice sites in patients 1 and 2 did not reveal additional nucleotide variation predicting an amino acid change or a splicing abnormality. Complete sequencing of introns 73, 86, and 87 showed a normal sequence.

IF of Skin Biopsies

IF labeling of skin biopsies with LH7:2 antibody showed bright and linear staining along the dermo-epidermal junction in both patients, similar to what was seen in the control. Type IV collagen, laminin *5*, and bullous pemphigoid antigens were expressed normally.

Accumulation of Type VII Procollagen in Keratinocytes in Vitro

IF staining with antibodies to type VII collagen demonstrated a rather weak intracellular granular staining in normal control keratinocytes (fig. 4*a* and *c*). In contrast, the DDEB keratinocytes of both patients revealed a clearly increased intracellular type VII collagen content, as evidenced by a stronger IF signal (fig. 4*b* and *d*). Use of different domain-specific antibodies established that the material retained consisted of entire type VII procollagen molecules, containing the NC-1, NC-2, and triple-helical domains. Immunoblotting of keratinocyte extracts confirmed the visual impression derived from IF staining (fig. 5*a*). A single 320-kD band representing the proα1(VII) polypeptide chain was found in control and DEB cell media and extracts, but quantitative differences emerged. The type VII procollagen bands derived from keratinocytes of patients 1 and 2 were stronger than those from controls. A semiquantitative immunoblot assay using soluble chromophores and spectrophotometric determination of the immunoblot signal revealed that the content of type VII procollagen was higher in DDEB cells than in controls, indicating intracellular accumulation of type VII procollagen. When the signal intensity for controls was set at 1.0, it was 1.7 in cells of patient 1 and was 2.3 in cells of patient 2 (fig. 5*b*).

Pepsin Digestion of Mutant Type VII Procollagen

To allow investigation for the presence of the mutant type VII procollagen molecules in the patient's keratinocytes, the cell extracts were subjected to a limited pepsin digestion, a treatment that eliminates the globular domains of collagens and that leaves the pepsin-resistant triple-helical fragments undigested (Bruckner and Prockop 1981). This proteolytic fragmentation was necessary, since small deletions such as those in the present probands cannot be discerned, by SDS-PAGE, in a polypeptide with a size >300 kD (fig. 6, *left panel*). Since type VII collagen is very sensitive to proteolysis, a low enzyme concentration and a low reaction temperature were chosen for the pepsin treatment. Under these conditions, the globular NC-1 and NC-2 domains were removed from type VII procollagen. In contrast, the triple-helical domain mostly resisted digestion, except for a partial cleavage, into P1 and P2 fragments, at the site

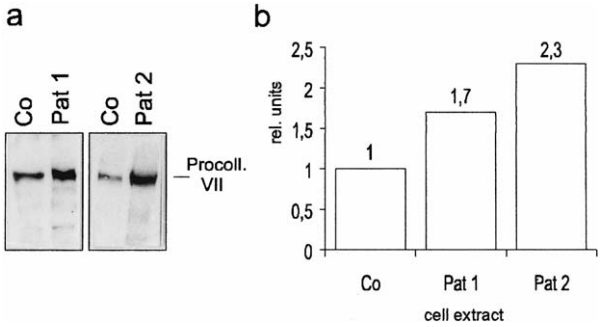

Figure 5 Immunoblotting of type VII collagen from control and patient's keratinocyte extracts. *a*,Intensity of 320-kD type VII procollagen immunoblot band, which was stronger in keratinocyte extracts of patients 1 (lane Pat 1) and 2 (lane Pat 2) than in those of controls (lanes Co). *b,* Semiquantitative immunoblot-based assay (König and Bruckner-Tuderman 1992), demonstrating that the relative intensity of type VII procollagen bands in the control, patient 1, and patient 2 was 1:1.7:2.3, indicating that the DDEB keratinocytes accumulated type VII procollagen.

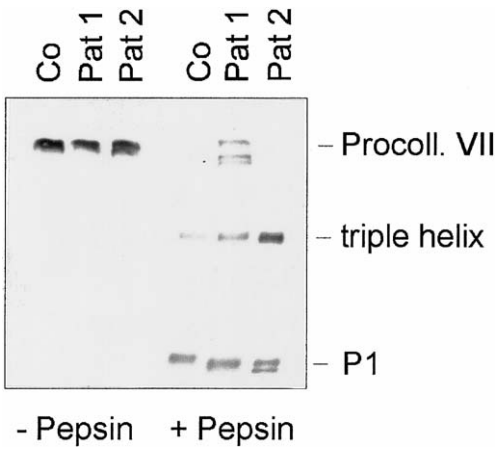

Figure 6 Pepsin digestion of type VII procollagen, and immunoblotting with antibodies to the carboxy-terminus of type VII collagen. Normal and DDEB keratinocyte extracts were digested with pepsin, to produce smaller triple-helical type VII procollagen fragments. In untreated extracts of the control and patient 1 and 2, the 320-kD type VII procollagen band was present (lanes −Pepsin). A limited digestion with pepsin (lanes +Pepsin) resulted in the loss of the intact procollagen and in appearance of the full-length triple helix and its carboxy-terminal half, the P1 fragment (Burgeson 1993). The amino-terminal half of the triple helix, the P2 fragment, was not recognized by the antibodies used here. In contrast to what was seen in the control with a distinct P1 fragment, P1 derived from mutant type VII procollagen of patient 1 appeared as a diffuse and broadened band, and pepsin digestion of mutant type VII procollagen of patient 2 resulted in a double P1 band, indicating that the mutant type VII collagen molecules were hybrid trimers of normal length and shortened polypeptides. In the case of patient 1, pepsin treatment did not result in complete digestion of the procollagen molecules but produced abnormal high–molecular-weight cleavage products, suggesting that inclusion of mutant polypeptides in the trimeric molecules had led to altered proteinase sensitivity.

of the hinge region, located in the center of the triple-helical domain, as has been described by Burgeson (1993). In controls, the fragment pattern described above was obtained (fig. 6, *right panel*). In case of patient 1, pepsin treatment yielded a diffuse band of the C-terminal P1 fragment, and fragmentation of type VII procollagen of patient 2 resulted in a distinct double band of the P1 fragment (fig. 6, *right panel*). These bands reflected the presence of normal and shorter, mutant proα1(VII) polypeptides within the triple helices of type VII procollagen in the patients. In addition, high-molecular-weight cleavage products were observed in patient 1, suggestive of altered proteinase sensitivity of the mutated molecule (fig. 6, *right panel*).

The different pattern of the P1 fragment in patient 1 versus that in patient 2 likely reflects the location of the deletion within the P1 fragment, with respect to the hinge region. Patient 1's deletion causes the replacement of 11 Gly-X-Y triplets adjacent to the hinge region by three amino acids, E-S-L, which disrupt a continuous stretch of 24 glycine triplet repeats. The mutated sequence of the region C-terminal from the hinge region now reads as follows: (G-X-Y)14-L-(G-X-Y)2 -E-S-L-(G-X-Y)11-VSVDEPGP-(G-X-Y)38. This sequence contains short Gly-X-Y repeats and multiple interruptions. It therefore is predicted to be more sensitive to pepsin, which would cleave this segment at several sites. As a result, several P1 fragments of slightly different size appear as a diffuse band on SDS-PAGE in patient 1.

Also, the fact that variable amounts of high-molecular-weight cleavage products constantly were observed with collagen VII from patient 1 supports the notion of altered proteolytic sensitivity of the mutated region, which presumably is due to altered protein folding at this site. In contrast, the deletion in patient 2 is located further C-terminally, at amino acid positions 2278–2300. This deletion resides in the middle of a long uninterrupted triple-helical sequence. Therefore, pepsin digestion of the hinge region in patient 2 produces a distinct double band, representing both a normal and a deleted P1 fragment.

The upper, normal P1 band from the patients appears to migrate somewhat faster than that in the control (fig. 6, *right panel*). This was a constant observation with the probands' P1 fragment. The reasons for this faster-migrating pattern remain unclear, but they may involve alterations in posttranslational modifications. However, very little is known about prolyl and lysyl hydroxylation or glycosylation of nascent proα1(VII) chains in keratinocytes. The normal P1 fragment contains 91 proline, 105 hydroxyproline, 9 lysine, and 41 hydroxylysine residues/1,000 amino acids (Morris et al. 1986), and it is conceivable that the trimeric molecules containing both normal and mutated chains are not adequately modified and that, therefore, they migrate faster.

AF Abnormalities

Electron microscopy of skin biopsies showed both cleavage beneath the lamina densa and alterations of AFs, in both patients. Patient 1 displayed a reduction in the number of AFs, which were thin and hypoplastic (fig. 7*b*). Patient 2 demonstrated rare AFs, which showed a morphology rudimentary (fig. 7*c*) in comparison with that in the control (fig. 7*a*).

Discussion

We have described two patients affected with DDEB who are heterozygous for genomic deletions within exons 73 and 87 in the collagenous domain of COL7A1. Both mutations alter splicing of the corresponding exon, despite intact consensus splice-site sequences. In patient 1, the 6081del28 mutation is associated with activation of a cryptic splice site within exon 73, and the combi-

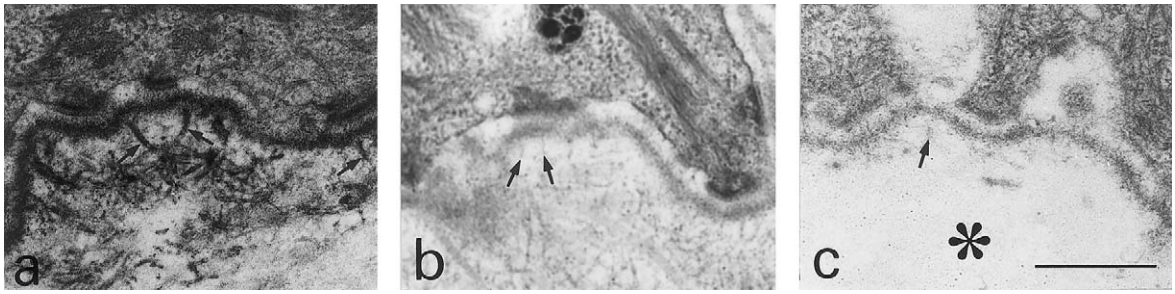

Figure 7 Electron microscopy of tissue of (*a*) the control, which shows AFs (*arrows*) of normal length and ultrastructure that insert into the lamina densa, (*b*) skin from patient 1, which shows hypoplastic AFs reduced in number, and (*c*) patient 2, which shows tissue separation (*asterisk* [*]) below the lamina densa and rare and poorly formed AFs. (Scale bar 0.5 μm; final magnification 26,400 ×)

nation of both defects results in the replacement of the last 10 Gly-X-Y repeats from this exon by a noncollagenous Glu-Ser-Leu sequence. In patient 2, the 6847del27 mutation causes in-frame skipping of exon 87 encoding 7 Gly-X-Y repeats and amino acid residues Gly2299 and Gln2300. This first report of COL7A1 deletions in DDEB suggests different possible mechanisms for abnormal splicing of exons 73 and 87 and indicates that shortened type VII collagen polypeptides can cause DDEB.

Proposed Mechanism Producing 6081del28 and 6847del27 Mutations

Analysis of the 6081del28 mutation identified in patient 1 shows that the 28-bp deleted sequence contains two inverted repeats—CCGGC/GCCGG and GGC/GCC—which could have potentiated the formation of a single-stranded hairpin loop (fig. 8*a*). In addition, examination of the sequence in the vicinity of the deletion shows two stretches—of 4 G residues and 6 C residues—immediately upstream of the deletion. Although the role that these stretches of repetitive residues may have in the generation of the deletion remains speculative, it is possible that they may have favored slippage of the DNA polymerase across the base of the loop during DNA replication and have led to skipping of the 28-bp sequence participating in the hairpin structure. Alternatively, the deletion may have arise from excision of the loop during DNA replication, as has been described elsewhere for transposable elements (McConkey 1995).

In patient 2, analysis of the 6847del27 mutation shows the presence of a CCTGGCCC repeated sequence (underlined sequence in fig. 8*b*) located at the 5′ end of the deleted sequence and immediately adjacent to its 3′ end. This sequence may have favored accidental nucleotide pairing, during DNA replication, and removal of the intervening sequence, corresponding to the deletion of nucleotides 6847–6873, through a slipped-strand–mispairing mechanism (Krawczak and Cooper 1991).

A Role for Exons 73 and 87 Sequences In Splice-Site Selection

In the absence of any other defect in the sequence of exons and introns 73, 86, and 87, including their putative branch sites, we suggest that abnormal splicing of exons 73 and 87 results from alteration in the sequence,

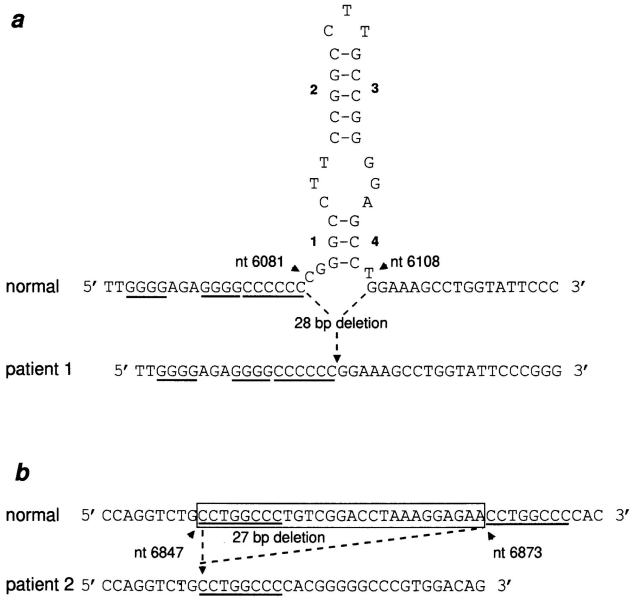

Figure 8 Proposed mechanisms for genomic deletions 6081del28 and 6847del27. *a*, Patient 1 (6081del28). Formation of a hairpin loop by intrastrand pairing of inverted repeats 1-4 and 2-3 is shown. The presence of stretches of G and C (*underlined*) located immediately upstream of the loop would have favored slippage of the DNA polymerase and replication across the base of the loop, causing the loop to be removed from the intervening sequence. Alternatively, excision of the loop may have occurred during DNA replication. *b*, Patient 2 (6847del27). The presence of an 8-bp direct repeat (CCTGGCCC [*underlined*]) may have favored accidental nucleotide pairing and deletion of the intervening 27-bp sequence during DNA replication.

size, and/or secondary structure of these exons, caused by 6081del28 and 6847del27.

Since mutations in ESEs recently have been reported in several disease genes reviewed by Cooper and Mattox (1997), we examined the possibility that such sequences could be altered by the mutations that we have described here. Analysis of the content, in purines and AC, of the present deletions (42% for 6081del28 and 55% for 6847del27, the AC content being similar to the purine content in each deletion) suggests that it is unlikely that they are ESE or ACE sequences. However, there is currently no consensus sequence for ESE motifs, and we cannot exclude the possibility that the partial deletions of exon 73 and 87 alter previously unidentified regulatory regions involved in splice-site selection.

Other exonic mutations that affect splicing, despite the presence of normal consensus splice sites, include nonsense mutations, the mechanism of action of which remains unclear (Dietz et al. 1993), and large deletions or insertions changing the size and/or secondary structure of the exon (Eperon et al. 1988; Matsuo et al. 1992; Narita et al. 1993). Skipping of exons containing nonsense mutations has been reported in a number of disease genes and is thought to involve a mechanism scanning for maintenance of an open reading frame (Dietz and Kendzior 1994). Abnormal splicing of exon 73 restores the phase, suggesting that a similar mechanism possibly could be involved. However, the 6081del28 mutation does not create a PTC in exon 73 but does do so in exon 82. Therefore, it remains uncertain whether the open-reading-frame interruption caused by the 6081del28 mutation might contribute to abnormal splicing of exon 73. In contrast, the 6847del27 mutation maintains the reading frame, and thus it is not plausible that skipping of exon 87 involves a termination-codon–recognition mechanism.

On the other hand, the reduction in size of exon 73 (from 201 to 173 bp) and of exon 87 (from 69 to 42 bp) alters the distance between the 3′ and 5′ splice junctions of these exons. This distance has been shown, in other genes, to be critical for "exon definition," in the sense that interacting splicing factors may require an optimal interaction range across the exon (Reed 1996). In particular, reduction of the size of exons to <50 bp has been shown to lead to exon skipping (Dominski and Kole 1991; Berget 1995). In addition, small exons (<60 bp) have been noted to have strong splice sites to prevent them from being skipped (Zhang 1998). Therefore, we calculated the scores for the splice-site sequences flanking exon 87, as described by Shapiro and Senapathy (1987). We found a score of .61 for positions −6 to +2 of the splice acceptor sequence of intron 86 and a score of .63 for positions −2 to +6 of the splice donor sequence of intron 87. These relatively low values suggest that shortening of exon 87 would have hindered recognition of this exon and its flanking splice sites during spliceosome assembly and would have favored the use of the stronger acceptor splice site of intron 87 (score .84), leading to skipping of exon 87.

Finally, it is also possible that the removal of genomic sequences disrupts the pre-mRNA secondary structure of exons 73 and 87 that is important for splicing. In support of this possibility, 6081del28 eliminates a hairpin from the predicted mRNA secondary structure of exon 73 (obtained by use of the "Mfold" computer program, for RNA, by Zuker (1994).

Mechanism for Abnormal AF Formation

The two deletions that we have described here result in the synthesis of mutated type VII polypeptides that would be 30 and 23 amino acid residues shorter than the wild-type chains. In addition, a short noncollagenous sequence is introduced into exon 73 in patient 1. By analogy with other single-exon deletions in COL1A2, COL3A1, and COL11A2 genes—reported in osteogenesis imperfecta (OI) type I (Zhuang et al. 1993), Ehlers-Danlos syndrome type IV (Kuivaniemi et al. 1995), and dominant Stickler syndrome (Vikkula et al. 1995), respectively—we suggest that these structurally abnormal polypeptides associate with their normal partner and impair folding of the triple helix in a dominant manner. The alteration of protein conformation would account for delayed secretion and intracellular accumulation of type VII collagen molecules, as observed in culture keratinocytes of the probands. In patient 1, the replacement of the last 11 Gly-X-Y repeats of exon 73 by a Glu-Ser-Leu sequence creates a segment of short Gly-X-Y repeats and multiple interruptions adjacent to the hinge region. In patient 2, skipping of exon 87 disrupts a continuous stretch of 28 Gly-X-Y repeats in the 14th collagenous subdomain. In both cases, shortening of the collagenous domains creates a shift in the alignment of Gly-X-Y repeats in one chain relative to the other, in trimeric hybrid molecules. In addition, although the center of the helix accommodates only residues of glycine, the smallest amino acid, in patient 1 the noncollagenous Glu-Ser-Leu sequence introduces both a bulky and charged residue (Glu) into the center of the triple helix, which may establish charge-charge interactions with neighboring amino acid residues. We suggest that the reduction of normal type VII collagen homotrimers and dominant-negative effects of hybrid molecules containing mutated chains would impair AF formation and cause loss of dermal-epidermal adhesion in heterozygotes.

Because of both poor solubility and sensitivity to unspecific degradation of procollagen VII in the medium during biochemical manipulations (Burgeson et al. 1990; König and Bruckner-Tuderman 1992), the ratio of normal and mutated molecules secreted by the patients' cells

could not be determined reliably. However, the paucity and abnormal morphology of the AFs in the patients' skin support the assumption that some mutant molecules are secreted and interfere with normal AF formation in a dominant-negative manner. Similarly, studies of mutated collagen I in OI have demonstrated that not only normal but also some mutated collagen molecules are secreted into the medium and contribute to the OI phenotype, via dominant-negative interference with fibril formation (Raghunath et al. 1994).

Genotype-Phenotype Correlations

The COL7A1 defects that we have described here have been identified in DDEB patients whose skin-blistering condition had shown gradually improvement during childhood and almost had ceased by the onset of adulthood. Interestingly, a COL7A1 splice-site mutation that had been predicted to cause in-frame skipping of exon 36 recently has been described in dominantly inherited transient bullous dermolysis of the newborn (Christiano et al. 1997*b*). However, both the persistence, in the families that we have described, of blistering during late childhood and the existence of AF abnormalities at adulthood argue against the possibility that these patients could be affected with transient bullous dermolysis of the newborn. Considerable intrafamilial phenotypic variability was found in family 2, in which pretibial blistering was noted in the proband only and did not occur in his affected grandmother and father. The reasons for these observations remain unclear, but they suggest that other genetic determinants and/or environmental factors could influence the phenotypic outcome of the mutations. In this respect, it is possible that differences in the expression of the mutated allele and/or in the degradation of the abnormally folded protein could modulate the severity of the disease (Ashkenas and Byers 1997). Finally, one can speculate that in-frame removal of collagenous sequences could underlie DEB phenotypes of differing severity and modes of inheritance, as is the case with glycine mutations in COL7A1 that cause DDEB or RDEB.

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Genbank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for accession numbers L02870 and L23982)
Mfold, http://www.ibc.wustl.edu/~zuker) (for prediction of RNA secondary structure)

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