Partial Revertant Mosaicism of Keratin 14 in a Patient with Recessive Epidermolysis Bullosa Simplex[1]

Petra H. L. Schuilenga-Hut, Hans Scheffer,[2] Hendri H. Pas,* Miranda Nijenhuis,* Charles H. C. M. Buys, and Marcel F. Jonkman*

Department of Medical Genetics, University of Groningen, Groningen, the Netherlands; *Center for Bullous Skin Diseases, Department of Dermatology, Groningen University Hospital, Groningen, the Netherlands

A patient with recessive epidermolysis bullosa simplex due to a previously described homozygous *KRT14* 1842–2A→C splice-site mutation was re-examined, because we unexpectedly found signs of revertant mosaicism. The germline mutation resulted in different aberrant transcripts containing premature termination codons, all leading to truncated keratin 14 proteins. Basal keratinocytes in skin and in culture completely lacked keratin 14 and intermediate filaments. From this keratin $14^{-/-}$ patient we started cultures from a new skin biopsy and here, we serendipitously found keratinocytes that spontaneously expressed keratin 14. This biopsy had been taken from an area of skin that was clinically affected, because blisters could simply be evoked by gentle rubbing. Immunofluorescence and electron microscopy of additional biopsies from this skin area revealed a mosaic expression of keratin 14 and reappearance of intermediate filaments in basal keratinocytes. Immunoblotting showed a revertant keratin 14 polypeptide with seemingly normal molecular weight. DNA analysis of exon 2 and its flanking intron borders showed no additional mutations in the genomic *KRT14* sequence. Analysis of mRNA isolated from mosaic skin keratinocytes revealed an additional in-frame transcript (1844T→G, 1845Δ6) that codes for an abnormal keratin 14 polypeptide with a two residue deletion and one amino acid change. The re-expression of a revertant, albeit abnormal, keratin 14 polypeptide, so-called partial revertant mosaicism, accounts for the antibody staining pattern and for the reappearance of intermediate filaments, which however, are semifunctional and not able to revert the clinical phenotype. The combination of a keratin 14-positive and a keratin 14-negative cell population in epidermis as well as in cultured keratinocytes suggests that the cellular reversion might be caused by an endogenous factor. We hypothesize that a second somatic modulating factor in the genome that affects the processing of the mutant *KRT14* pre-mRNA may underlie this phenomenon. ***Key words: cytoskeleton/gene reversion/mosaicism/tonofilaments. J Invest Dermatol 118:626–630, 2002***

Revertant mosaicism in skin is a new genetic principle that has been discovered recently (Jonkman *et al*, 1997), for review (Jonkman, 1999). A revertant is a mutant that has regained, partially or completely, the wild-type phenotype by either genetic or nongenetic mechanism of reversion (Rieger *et al*, 1996). Partial revertant mosaicism is a phenomenon in which the reversion is partial, for instance, in which the revertant protein is immunohistochemical reactive, but does not function, thus not leading to clinical improvement.

Manuscript received June 26, 2001; revised October 29, 2001; accepted for publication November 5, 2001.

Reprint requests to: Dr. Marcel F. Jonkman, Department of Dermatology, Groningen University Hospital, Hanzeplein 1, NL-9700 RB Groningen, the Netherlands. Email: m.f.jonkman@derm.azg.nl

Abbreviations: REBS, recessive epidermolysis bullosa simplex.

[1]Part of this paper was presented at the 2nd Scientific Meeting of the Dutch Society for Experimental Dermatology, Lunteren, the Netherlands, February 8, 2001 and on the 30th Annual Meeting of the European Society for Dermatological Research, Berlin, September 22, 2000.

[2]Currently appointed to Section DNA Diagnostics, University Medical Center Nijmegen St Radboud, Nijmegen, the Netherlands.

In this study we report a patient with keratin 14 $(K14)^{-/-}$ recessive epidermolysis bullosa simplex (REBS) who was a partial revertant mosaic. During gene correction experiments (Van der Steege *et al*, 2001) we serendipitously found keratinocytes that spontaneously expressed K14 in cell cultures derived from the $K14^{-/-}$ patient. Upon immunofluorescence examination of a new skin biopsy, K14 appeared to be present in the epidermis in a mosaic pattern. In an attempt to disclose the underlying cause of this revertant mosaicism, the patient was re-examined clinically, and the keratinocytes from revertant and mutant skin were analyzed at the DNA, RNA, and protein level.

MATERIAL AND METHODS

Patient characteristics and skin biopsies The patient was a female, age 67 y, suffering from REBS, previously reported by us as patient III-2 (Jonkman *et al*, 1996). On the genomic DNA level, a homozygous *KRT14*: 1842–2A→C transition was detected, situated in the acceptor splice site of intron 1. In the initial study, two aberrant mRNA transcripts generated by this mutation were detected, including one with complete skipping of exon 2, and one containing a deletion of the first 10 bp of exon 2 (Jonkman *et al*, 1996). This null mutation resulted in the complete ablation of K14 and of tonofilaments in basal cells. The revertant keratinocytes described in this study originated from a biopsy of the lower

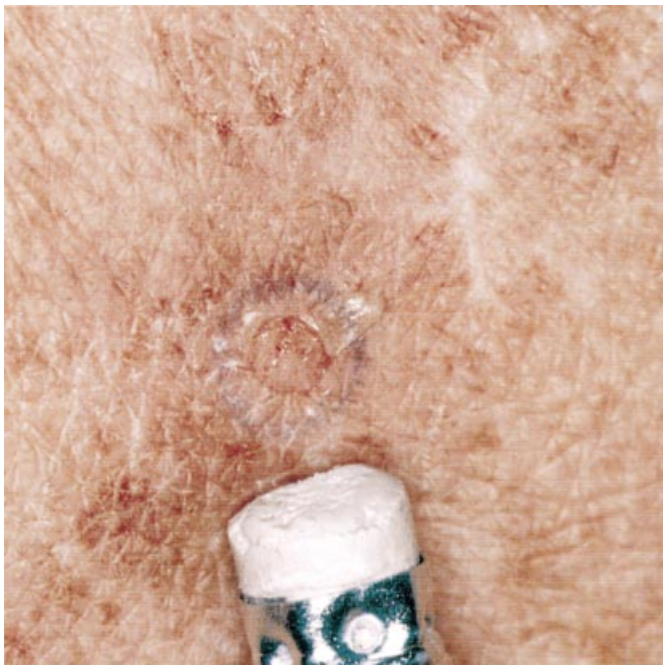

Figure 1. Erosion could easily be evoked on revertant skin. The skin of the right lower arm after rubbing with the eraser of a pencil. The scar of the previous biopsy is visible in the upper right corner.

right arm of the patient. Two more biopsies were taken from K14-negative skin on the trunk. In addition we re-examined the skin biopsies of other affected individuals of this family and of two unrelated REBS patients with the same mutation (Hut *et al*, 2000).

Keratinocytes were harvested from biopsies taken from the revertant and mutant skin, and cultured under serum-free conditions. A skin biopsy sample of an unaffected individual was included for reference.

Immunofluorescence microscopy For immunofluorescence microscopy on tissue samples, unfixed cryostat sections of snap frozen skin biopsies were processed as previously described (Jonkman *et al*, 1996). K14 was stained with monoclonal antibodies LL001 and LL002 (kind gift of Prof. B. Lane, Dundee) directed against the carboxy-terminal tail of the protein (Purkis *et al*, 1999). K5 + 8 was stained with monoclonal antibody RCK102 (kind gift of Dr. F.C.S. Ramaekers, Maastricht). As a secondary step we used Alexa488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR).

For immunofluorescence on cultured keratinocytes, cells were grown on glass coverslips, washed with phosphate-buffered saline (PBS) and fixed for 10 min with 1% formaldehyde in PBS. Cells were permeabilized by incubation in 0.5% Triton X-100 in PBS for 5 min, followed by blocking with 1% (wt/vol) bovine serum albumin in PBS for 20 min at 37°C. Subsequent incubation with primary antibody was carried out for 45 min at 37°C. The cells were rinsed three times with PBS and incubated with Alexa488-labeled goat anti-mouse IgG for 30 min at room temperature. Finally, the nuclei were stained blue with bisbenzamide.

Electron microscopy was performed as described previously (Jonkman *et al*, 1996).

Immunoblotting Preparation of cell extracts and immunoblotting was as described before (Pas *et al*, 1995). K14-specific monoclonal antibody was LL002. Bound antibody was detected by subsequent incubation with goat anti-mouse IgG followed by alkaline phosphatase-conjugated rabbit anti-goat IgG.

Mutation analysis DNA was isolated from primary keratinocytes by high salt/chloroform extraction (Miller *et al*, 1988). Prior to polymerase chain reaction (PCR) amplification of *KRT14* exon 2, the DNA was digested with *Alu*I in order to prevent coamplification of the highly homologous *KRT14* pseudogene (Savtchenko *et al*, 1988) as described previously (Hut *et al*, 2000). After amplification the PCR products were purified using the high pure PCR product purification kit (Boehringer Mannheim, Basel, Switzerland), and sequenced by automated DNA sequencing (ABI 377, Perkin Elmer, Norwalk, CT).

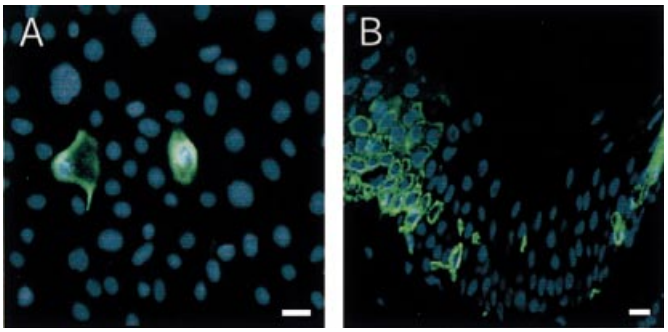

Figure 2. Mosaic expression of K14 in keratinocyte culture and in epidermis. (*A*) Keratinocyte culture; (*B*) epidermis.

Haplotyping To investigate if the patient had inherited *KRT14* alleles that were identical by descent to the *KRT14* alleles carrying the *KRT14*: 1842–2A→C splice site mutation of the previously described REBS patients (Jonkman *et al*, 1996; Hut *et al*, 2000), three microsatellite markers were analyzed flanking the *KRT14* locus, namely D17S800, D17S1814, and D17S1851. *KRT14* is located between D17S800 and D17S1818 (Genemap 1999, http://www.ncbi.nlm.nih.gov/genemap/). D17S1814 genetically maps to the same position as D17S1818. Therefore, we conclude that the most likely order of these loci is D17S800–*KRT14*–D17S1814–D17S1851. DNA was extracted from peripheral blood by the high salt/chloroform extraction method. To amplify the particular sequences, the following PCR program was used: denaturation at 94°C for 1 min, annealing at 56°C (D17S800 and D17S1851) or 62°C (D17S1814) for 1 min, and extension at 72°C for 1 min (30 cycles). All forward primers were fluorescein labeled. The lengths of the PCR products were determined using an automated sequencer (ALF, Amersham Pharmacia, Biotech AB, Uppsala, Sweden).

Analysis of RNA splice variants RNA was extracted from keratinocyte cultures by the RNeasy Mini Kit (QIAGEN Genomics Inc., Bothell, Washington). The Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and random primers were used for the synthesis of first-strand cDNA. After cDNA synthesis, a reverse transcription–PCR was carried out with the forward primer 5′-GAGAAGGTGACCATGCAGAA-3′ and the reverse primer 5′-ACATTGACATCTCCACCCAC-3′ (nucleotide positions 403–3022). For each sample 10 μl cDNA, 10 μl reaction buffer (with a final concentration of 10 mM Tris–HCl pH 9, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% wt/vol gelatin and 0.1% vol/vol Triton X-100), 40 pmol forward primer, 40 pmol reverse primer, and 2.5 units rTaq polymerase (Pharmacia Biotech, Piscataway, NJ) were used in a total volume of 100 μl. The amplicons were inserted into the pCRII cloning vector (Invitrogen, San Diego, CA) and transformed to *Escherichia coli* strain INVaF′. After spreading the culture on to NZYM plates containing ampicillin and X-gal, several white colonies of each sample were cultured, plasmids were isolated and subjected to direct sequencing using an automated DNA sequencer (ABI 377, Perkin Elmer).

RESULTS

Clinical phenotype was not reverted The skin of the patient was generalized affected; after meticulous inspection we could not find any areas of skin with signs of clinical reversion (**Fig 1**).

Cellular phenotype was reverted Immunofluorescence analysis of cultured keratinocytes derived from the right lower arm revealed a mixture of K14-positive and K14-negative keratinocytes (**Fig 2*A***), whereas in skin biopsies from other areas only K14-negative cells could be detected. Immunofluorescence analysis of a skin biopsy from the same area revealed an epidermis with a mosaic K14 pattern with clusters of K14-negative and K14-positive keratinocytes (**Fig 2*B***). The epidermal staining pattern of K5 was normal and was not altered by K14 mosaicism (data not shown).

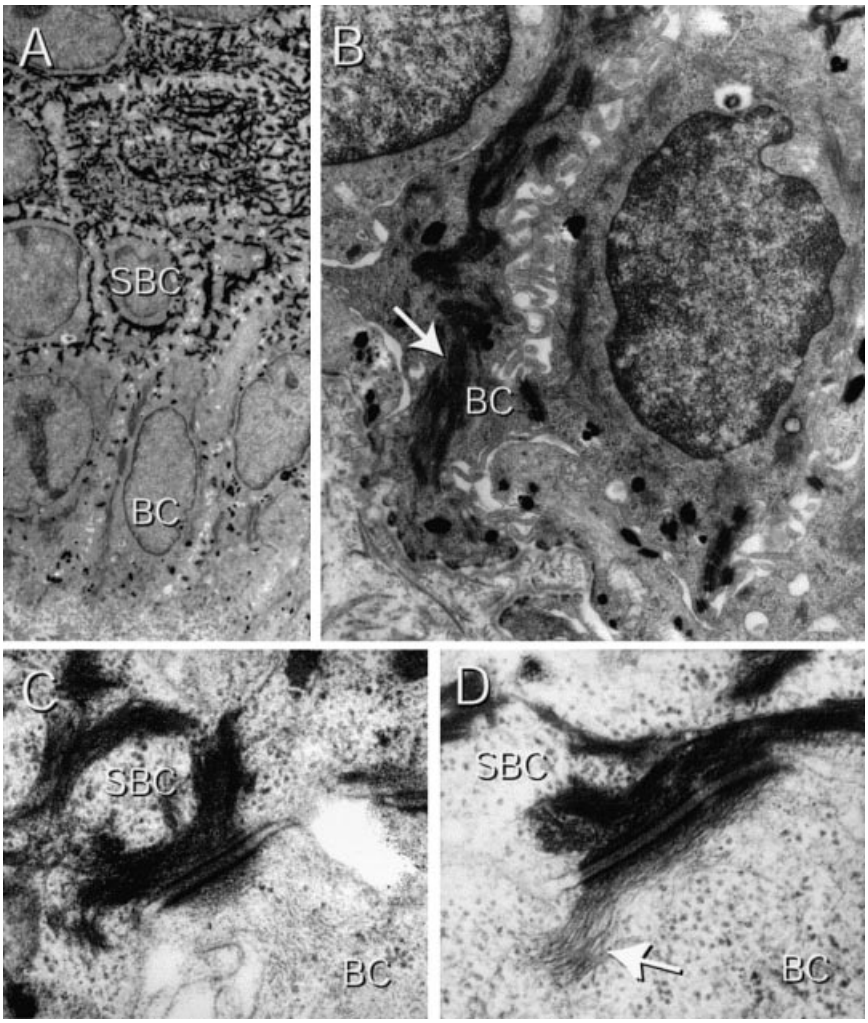

Figure 3. Tonofilaments were expressed in revertant basal keratinocytes. Tonofilament bundles (*arrows*) were re-expressed, although reduced, in basal cells of revertant skin (*A*, overview, and *B*, detail). *Panels C* and *D* show desmosomes between basal (BC) and suprabasal cells (SBC). In mutant skin (C) the tonofilaments were lacking in the basal cell, whereas in revertant skin (*D*) a reduced amount of tonofilaments inserted into the desmosome.

Electron microscopy showed reduced reappearance of tonofilaments in basal cells of revertant skin (**Fig 3**). The revertant tonofilament bundles were abnormally packed similar as in dominant EBS Köbner.

By immunoblot analysis, the production of K14 molecules appeared to be partially restored in revertant keratinocytes (**Fig 4**). The revertant K14 molecules apparently had the same mobility as wild-type K14 of ≈ 50 kDa.

Revertant and mutant keratinocytes were genetically similar at the *KRT14* locus Both negative and revertant keratinocytes contained the original *KRT14*: 1842–2A→C splice-site mutation at the junction of intron 1 and exon 2 (Hut *et al*, 2000). No additional mutation in exon 2 and its flanking intron borders was identified in the genomic *KRT14* sequence.

Haplotype analysis of the chromosomal region 17q harboring the *KRT14* locus in the REBS patients demonstrated that all three affected family members were homozygous for the analyzed polymorphic markers. When the data of the two other, unrelated, REBS patients were included, we found that at least for D17S800 all mutant alleles were identical, whereas one allele differed in D17S1814, and two alleles differed in the more distant marker. This may indicate that the 1842–2A→C mutation has originally occurred on a (1,1,1) haplotype causing a founder effect in the Dutch population, and during subsequent meioses the shared segment from this haplotype has become smaller in some chromosomes of more distantly related patients (**Table I**).

Only revertant keratinocytes produced an aberrant in-frame *KRT14* transcript RNA analysis of mutant keratinocytes showed the two initially described non-sense RNA splice variants: one with skipping of exon 2 and a second one containing a 10 bp deletion. The revertant keratinocytes, however, contained the 10 bp deletion transcript and in addition a transcript harboring a 6 bp deletion in combination with a U to a G change (**Table II**). In contrast with the other transcripts, this Δ6 bp transcript did have an intact reading frame. We unexpectedly also found an additional transcript, which we traced back to the K14 pseudogene. This transcript was found in mutant and revertant keratinocytes of the patient, but not in wild-type keratinocytes of a normal individual (sequence data not shown).

DISCUSSION

In this study we describe a patient with the typical clinical features of REBS. Although this patient, at the genomic level, only had the homozygous splice site mutation 1842–2A→C, her skin had areas with, clinically unnoticeable, mosaic K14 expression.

Analysis of the *KRT14* transcripts in the K14-positive cells revealed the presence of an additional mRNA splice variant. This splice variant is characterized by an in-frame deletion of 6 bp and a

transversion of a U to a G both at the beginning of exon 2, whereas the other detected splice variants, Δexon 2 and Δ10 bp, led to an out-of-frame sequence. The in-frame splice variant results in a slightly altered K14 polypeptide with one amino acid substitution and a deletion of two amino acids. The in-frame skip was located at the beginning of the 1B helical rod domain (**Fig 5**) and will undoubtedly affect normal winding of the keratin α-helix.

The limited re-expression of aberrant K14 polypeptides in revertant keratinocytes explains the reactivity with K14 monoclonal antibodies and the reappearance of semifunctional intermediate filaments. In fact, the disease was altered from homozygous REBS to hemizygous EBS with expression of aberrant K14 polypeptides, which even might have resulted in dominant-negative EBS if wild-type K14 polypeptides had been coexpressed.

The dispersion of K14-positive cells was not diffuse, but clustered, both in skin and in cultured cells. Therefore, the presence of the in-frame transcript is most likely not due to either a random or an environmental factor, but to an endogenous factor, most likely a second somatic modulating factor in the genome that affects the processing of the mutant *KRT14* pre-mRNA. Further research will be necessary to elucidate the molecular mechanism underlying the reversion described here.

Patient keratinocytes also contained a pseudogene transcript. This K14 pseudogene originated from the functional *KRT14* gene during human evolution (Savtchenko *et al*, 1988), and was inactivated by three frame shifts in coding regions and three mutations interfering with mRNA processing signals such as intron/exon boundaries and the polyadenylation signal. In this study, remarkably, transcription of the K14 pseudogene was detected only in keratinocytes with a mutant *KRT14* and not in normal keratinocytes. An explanation is that the pseudogene was upregulated in mutant and revertant keratinocytes. More likely is

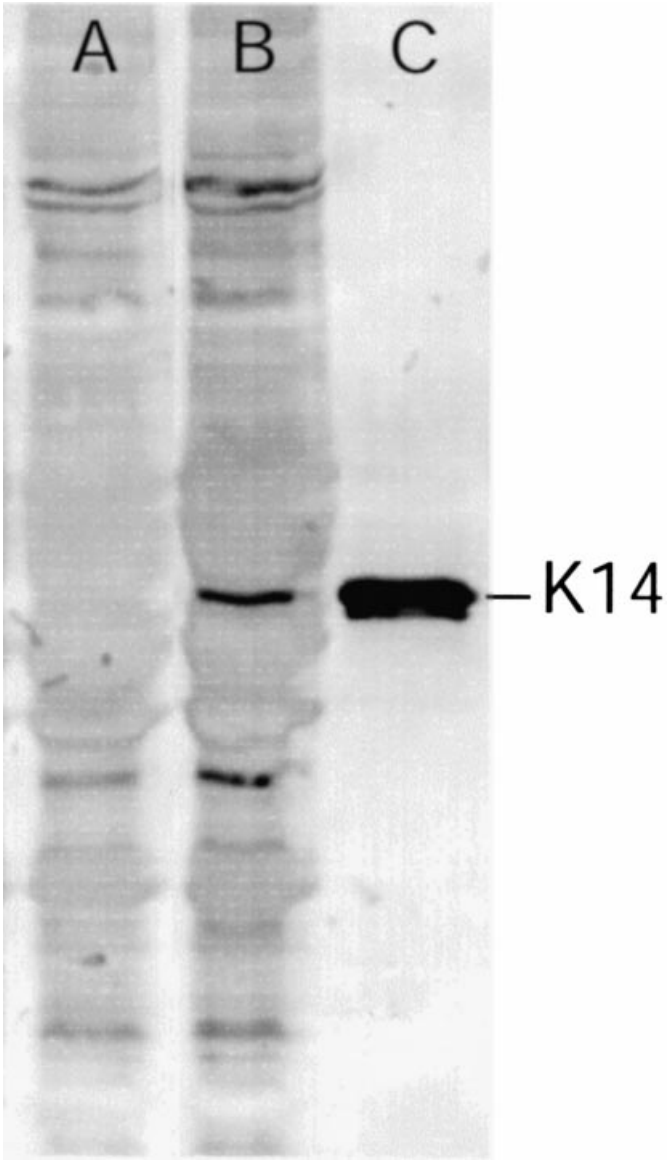

Figure 4. Revertant keratinocytes re-expressed K14. Immunoblot analysis of cell extracts of wild-type keratinocytes (*lane C*), keratinocytes obtained from a K14-negative skin (*lane A*) and keratinocytes obtained from a mosaic skin (*lane B*).

Table I. Results of *KRT14* haplotype analysis

Patients markers	Distance to top of chromosome 17	III-2[a]	III-8[a]	IV-30[a]	VII[b]	IX[b]
D17S800	62.9 cM	1 1	1 1	1 1	1 1	1 1
D17S1814	61.5 cM	1 1	1 1	1 1	1 1	1 2
D17S1851	61.0 cM	1 1	1 1	1 1	1 2	1 2

[a]Jonkman *et al* (1996).
[b]Hut *et al* (2000).

Table II. Clonal cDNA analysis of *KRT14*

	Δ10 bp	Δexon 2	T→G, Δ6	pseudogen	wild type
K14 mutant skin	5 (22)[a]	3 (13)	0	15 (65)	0
K14 revertant skin	2 (6)	0	9 (29)	20 (65)	0
normal human skin	0	0	0	0	25 (100)

[a]Percentage between brackets

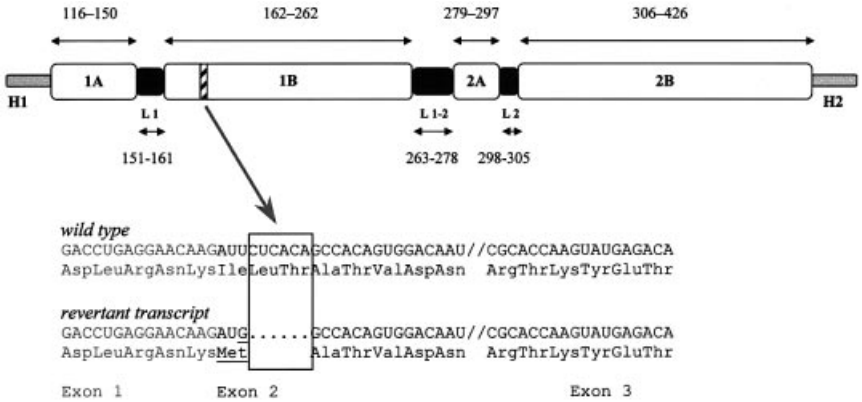

Figure 5. A schematic drawing of the K14 protein and the amino acid changes resulting from the revertant transcript. The striated region in the protein concerns the deleted amino acids 177 and 178 in the revertant protein. The numbering indicates the codon numbers according to Marchuk *et al* (1985).

that the relative account of K14 pseudogene transcripts in the total set of amplicons after K14 RNA reverse transcription–PCR is too low to be noticed in normal cells, but its ratio increases in the absence of wild-type transcripts in mutant and partial revertant cells.

Revertant mosaicism was first described in a type XVII collagen-deficient patient with generalized atrophic benign epidermolysis bullosa who was compound heterozygous for *COL17A1* null mutations, and showed complete reversal of a mutant allele to a normal sequence as the result of somatic gene conversion (Jonkman *et al*, 1997). Partial revertant mosaicism has been reported in another patient with generalized atrophic benign epidermolysis bullosa, who was homozygous for a two-nucleotide deletion in *COL17A1* (Darling *et al*, 1999). In one allele the reading frame was corrected by a downstream two-nucleotide insertion, thus this mosaic was due to a second locus mutation. The patient expressed aberrant type XVII collagen in a mosaic pattern, which did not have any clinical effect on the skin. In the partial revertant mosaic reported in this study the mosaicism is due to a third mechanism: alteration of mRNA by influencing the splicing process. The mechanisms underlying revertant mosaicism give insight into potential therapeutic intervention of genetic diseases.

We thank the patient for her collaboration. This study was supported by a grant from DEBRA UK.

REFERENCES

Darling TN, Yee C, Bauer JW, Hintner H, Yancey KB: Revertant mosaicism: partial correction of a germ-line mutation in COL17A1 by a frame-restoring mutation. *J Clin Invest* 103:1371–1377, 1999

Hut PHL, Vlies P, Jonkman MF, Verlind E, Shimizu H, Buys CHCM, Scheffer H: Exempting homologous pseudogene sequences from PCR amplification allows genomic keratin 14 hotspot mutation analysis. *J Invest Dermatol* 114:616–619, 2000

Jonkman MF: Revertant mosaicism in human genetic disorders. *Am J Med Genet* 85:361–364, 1999

Jonkman MF, Heeres K, Pas HH, *et al*. Effects of keratin 14 ablation on the clinical and cellular phenotype in a kindred with recessive epidermolysis bullosa simplex. *J Invest Dermatol* 107:764–769, 1996

Jonkman MF, Scheffer H, Stulp RP, *et al*. Revertant mosaicism in epidermolysis bullosa caused by mitotic gene conversion. *Cell* 88:543–551, 1997

Marchuk D, McCrohon S, Fuchs E: Complete sequence of a gene encoding a human type I keratin: sequences homologous to enhancer elements in the regulatory region of the gene. *Proc Natl Acad Sci USA* 82:1609–1613, 1985

Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215, 1988

Pas HH, De Jong MCJM, Jonkman MF, Heeres K, Slijper-Pal I, Van der Meer JB: Bullous pemphigoid: serum antibody titre and antigen specificity. *Exp Dermatol* 4:372–376, 1995

Purkis P, Steel JB, Mackenzie IC, Nathrath WBJ, Leigh IM, Lane EB: Antibody markers of basal cells in complex epithelia. *J Cell Sci* 97:39–50, 1999

Rieger R, Michaelis A, Green MM: *Glossary of Genetics and Cytogenetics: Classical and Molecular*, 4th edn. New York: Springer-Verlag, 1996: 478–478

Savtchenko ES, Freedberg IM, Choi IY, Blumenberg M: Inactivation of human keratin genes: the spectrum of mutations in the sequence of an acidic keratin pseudogene. *Mol Biol Evol* 5:97–108, 1988

Van der Steege G, Schuilenga-Hut PHL, Buys CHCM, Scheffer H, Pas HH, Jonkman MF: Persistent failures in gene repair. *Nat Biotechnol* 19:305–306, 2001