Transcriptional Activation of Endogenous Retroviral Sequences in Human Epidermal Keratinocytes by UVB Irradiation

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Ultraviolet radiation is a pathogenic factor in various diseases, e.g., autoimmune disorders such as lupus erythematosus. On the other hand, endogenous retroviruses are discussed as etiologic agents in lupus erythematosus. Therefore, we investigated the influence of ultraviolet irradiation on expression of human endogenous retroviral sequences and human endogenous retroviral sequence promoter-driven transcription of cellular genes using human epidermal keratinocytes as a model system. First, conserved sequences of endogenous retroviral *pol* genes were amplified from cellular mRNA by reverse transcriptase polymerase chain reaction with degenerate oligonucleotide primers. Polymerase chain reaction products were hybridized in a reverse dot blot hybridization assay to a representative number of distinct cloned human endogenous retroviral *pol* fragments. Using this method, we could show that irradiation with 30 mJ per cm^2 ultraviolet B activates transcription of various endogenous retroviral *pol* sequences in primary epidermal keratinocytes as well as in a spontaneously immortalized keratinocyte cell line (HaCaT). Interestingly, some of these sequences were found to be closely related to *pol* sequences of human endogenous retroviral sequences which have been shown to be expressed in autoimmune patients. Analysis of human endogenous retroviral *pol* expression *in vivo* using skin biopsies of lupus erythematosus patients revealed similar activation patterns. In a second approach, ultraviolet B- induced chimeric transcripts were isolated which are initiated by human endogenous retroviral promoters and proceed into cellular sequences using a newly established modified differential display polymerase chain reaction technique. The activation of human endogenous retroviral sequence transcription by ultraviolet B may contribute to the pathogenesis of lupus erythematosus, where inappropriate antigenic presentation of ultraviolet B-induced viral and cellular proteins could stimulate autoantibody production. ***Key words: autoantigens/differential display polymerase chain reaction/lupus erythematosus/reverse dot blot hybridization. J Invest Dermatol 113:587–594, 1999***

Endogenous retroviruses or retrovirus-like genetic elements are an integral part of the eukaryotic genome. They may be relics of former exogenous retroviruses which have been integrated into the DNA of germline cells and dispersed via retrotransposition during the course of evolution. It has been demonstrated that at least 2% of the human genome consists of these retrovirus-related sequences (reviewed by Leib-Mösch *et al*, 1990; Wilkinson *et al*, 1994; Leib-Mösch and Seifarth, 1995). The structure of human endogenous retroviral sequences (HERV) resembles exogenous retroviruses. Sequences encoding capsid proteins (Gag), reverse transcriptase (Pol), and envelope proteins (Env) are flanked by long-terminal repeats (LTR) containing the regulatory sequences necessary for retroviral transcription. Besides these flanking LTR, solitary HERV LTR are present in the human genome in very high copy numbers. Most HERV discovered so far are inactivated by frameshift mutations or deletions and are therefore replication defective. Some full-length HERV, however, contain all the structural features essential for viral gene expression and replication (Mayer *et al*, 1999). Indeed, transcriptional activity of HERV has been demonstrated in various human cells and tissues (reviewed in Wilkinson *et al*, 1994; Hohenadl *et al*, 1996; Löwer *et al*, 1996). Thus, HERV represent a reservoir of possibly pathogenic viral genes that may either be activated spontaneously or by environmental influences. With respect to environmental conditions there are some reports investigating the effects of ultraviolet (UV) radiation on viral infections or viruses themselves (reviewed by Norval *et al*, 1994). With regard to human retroviruses, it has been demonstrated that human immunodeficiency virus (HIV)-1 is activated by UVB

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Abbreviations: HERV, human endogenous retroviral sequence; LE, lupus erythematosus; LTR, long-terminal repeat; pol, polymerase.

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or UVC in a chronically infected promonocytic cell line (Stanley *et al*, 1989). UVC irradiation also increased HIV-directed gene expression in stably transfected cell lines via the viral LTR promoter (Valerie *et al*, 1995), and activation of a HIV-1 LTR-driven reporter gene in response to UVB irradiation was demonstrated *in vivo* in the skin of transgenic mice (Vogel *et al*, 1992). UV radiation is also well known to have deleterious effects on human skin and to play a pathogenic role in many different diseases, for example, in autoimmune disorders like lupus erythematosus (LE). Photosensitivity is a common feature within these patients and the typical skin lesions as well as systemic symptoms can be provoked by UV irradiation (Kind *et al*, 1993; Walchner *et al*, 1997). Furthermore, in the pathogenesis of autoimmune diseases an involvement of endogenous retroviruses is discussed (reviewed in Nakagawa and Harrison, 1996). Autoimmune diseases such as LE are mainly defined by the occurrence of autoantibodies reacting with self epitopes. Activated expression of HERV may lead to synthesis and immunologic presentation of retroviral proteins sharing amino acid sequence similarities with cellular proteins (molecular mimicry). In addition, the frequently described humoral immunity to exogenous retrovirus proteins in autoimmune patients could be explained by the existence of cross-reactive HERV proteins (Banki *et al*, 1992; Brookes *et al*, 1992; Perl *et al*, 1995).

In this study we first investigated the effects of UVA and UVB on the expression of HERV in human skin-derived keratinocytes. Transcriptional activation of a number of different HERV *pol* sequences in response to UVB irradiation could be demonstrated using a reverse dot blot hybridization assay. Similar HERV *pol* activation patterns were observed in UV-provoked or lesional skin areas of LE patients. Moreover, UVB-induced transcription of cellular sequences initiating within HERV LTR was detected by a newly established LTR-anchored differential display reverse transcriptase–polymerase chain reaction (DDRT–PCR).

MATERIALS AND METHODS

Cell culture Normal human epidermal keratinocytes (NHEK) were obtained from Clonetics Corp. (CellSystems, Remagen, Germany) and cultured according to supplier's instructions in keratinocyte growth medium supplemented with 0.1 ng per ml human recombinant epidermal growth factor (hEGF), 5.0 μg per ml insulin, 0.5 μg per ml hydrocortisone, 50 μg per ml gentamicin, 50 ng per ml amphotericin-B, 0.15 mM calcium, and 15 mg bovine pituitary extract (BPE). NHEK were seeded with a density of 2500 cells per cm^2, grown to 60–80% confluence, trypsinized (0.025% trypsin/0.01% EDTA), and re-established in culture. HaCaT cells (Boukamp *et al*, 1988) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and 4.5 mg per ml glucose.

UV irradiation Keratinocytes were grown to confluence in 10 cm culture dishes. Growth medium was removed and cells were washed twice with phosphate-buffered saline. UV radiation was applied with two different sources for UVB and UVA (UV 208 T, psoralen UVA 236 T, Waldmann Medizintechnik, Schwenningen, Germany) emitting a continuous spectrum between 280 and 370 nm (UVB) and 320–400 nm (UVA). UV lamps are covered with a 2 mm thick transparent polyacryl screen filtering out short wave UVC (<290 nm). Fluence rate at the site of irradiation was 0.79 mW per cm^2 for UVB and 12.2 mW per cm^2 for UVA as determined with an UV meter (Waldmann Medizintechnik, Schwenningen, Germany). Sublethal doses between 10 and 30 mJ per cm^2 UVB and 0.1–20 J per cm^2 UVA were applied by varying the exposure time. After UV treatment, cells were replenished with fresh culture medium and incubated for further 2 h, 6 h, and 24 h.

RNA isolation and cDNA synthesis. Cells were trypsinized, washed with phosphate-buffered saline and incubated for 30 s in 200 μl lysis buffer (10 mM Tris, pH 7.5, 140 mM NaCl, 5 mM KCl, 1% NP-40, 10 mM DTT, 0.4 U RNAsin (Promega, Heidelberg, Germany). Cell extracts were centrifuged (5 min, 925 × *g*) in an Eppendorf centrifuge and supernatants were collected. Cytoplasmic RNA was isolated after addition of 400 μl RLT buffer using the RNeasy Mini Total RNA Kit (Qiagen, Hilden, Germany) following supplier's instructions. About 1×10^7 cells were processed per RNeasy spin column. Poly(A)$^+$-RNA was isolated from purified total RNA with oligo(dT)-coated magnetic beads (Dynal, Hamburg, Germany) according to supplier's instructions. For cDNA synthesis 1 μg poly(A)$^+$ RNA was reverse transcribed in a 30 μl reaction using 150 pmol random hexamer primers, AMV reverse transcriptase (Boehringer, Mannheim, Germany) and the corresponding buffer. The reaction was performed at 42°C for 1 h and terminated by heating to 95°C for 5 min.

RNA isolation from skin biopsies Patients with different manifestations of LE (systemic or subacute cutaneous; see **Fig 2**) were included in this study after giving informed consent. Skin biopsies (4 mm in diameter) were obtained either from skin areas subjected to diagnostic UV phototesting (Walchner *et al*, 1997) from skin lesions or, as a control, from normal skin of the same patient. Biopsy material was homogenized in guanidinium thiocyanate buffer [4 M guanidine thiocyanate, 25 mM Na-citrate, 100 mM β-mercaptoethanol, 0.5% (vol/vol) sarcosyl; pH 7.0] and total RNA was isolated according to Chomczynski and Sacchi (1987). Poly(A)$^+$-RNA purification and cDNA synthesis was performed as described above.

Reverse dot blot hybridization Conserved sequences of the retroviral *pol* gene were amplified from cDNA using degenerate oligonucleotide primers modified according to Shih *et al* (1989) and Donehower *et al* (1990). The primers *RTfor* (5′-gatcggatccGT(AG)(CT)T(AGCT)CC(AGCT)CA(AG)GG-3′) and *RTrev* (5′-gatcaagctt(AG)TC(AG)T-C(AGCT)A(CT)(AG)TA-3′) were synthesized including recognition sites for *Bam*HI and *Hin*dIII, respectively (underlined). PCR was carried out in a 9600 thermal cycler (Perkin Elmer, Weiterstadt, Germany) with 80 pmol of each primer in a 100 μl reaction using the following cycle conditions: three cycles with 94°C, 30 s, 37°C, 1 min, 72°C, 1 min, and 30 cycles with 94°C, 30 s, 50°C, 1 min, 72°C, 1 min. PCR products were purified by agarose gel electrophoresis and reamplified with 50°C annealing temperature. For radioactive labeling of *pol* products, PCR was performed in the presence of 50 μCi α^{32}P-dCTP (3000 Ci per mmol, Amersham, Braunschweig, Germany) in a 40 μl reaction containing 250 μM dATP, dGTP, dTTP, and additionally 2.6 μM unlabeled dCTP. Separation of labeled products from primers and unincorporated nucleotides was achieved with a PCR purification kit (Qiagen, Hilden, Germany). Dot blots (Nytran-N membrane, Schleicher & Schuell, Dassel, Germany) carrying an ordered number of fragments of previously cloned and analyzed HERV *pol* sequences amplified from chromosomal DNA (Herrmann and Kalden, 1994) were hybridized with 2×10^6 c.p.m. per ml of denatured probe in 6 × sodium saline citrate (SSC) (900 mM NaCl, 90 mM sodium citrate, pH 7.0), 5 × Denhardt's, 30% formamide, 0.1% sodium dodecyl sulfate (SDS), and 100 μg per ml shared and denatured salmon sperm DNA for 16 h at 42°C. Filters were washed once in 2 × SSC/0.1% SDS for 15 min at room temperature, twice for 30 min at 50°C and finally once in 0.1 × SSC/0.1%SDS for 15 min at 65°C. Subsequently filters were exposed to Kodak X-Omat AR films.

LTR-anchored differential display PCR Synthesized cDNA was amplified by PCR using HERV-specific LTR primers (*LTR-K1*: 5′-CTGGCGGGATCCTCCATATGCTGAA-3′ or *LTR-H1*: 5′-TTG-GACTCAGCCCACCTGCAGCCAGG-3′) identical to a conserved sequence within the U5 region of HERV-K (Ono *et al*, 1986; Leib-Mösch *et al*, 1993) and the R-U5 region of HERV-H LTR (Goodchild *et al*, 1993), respectively. *ArbPrim1* (10mer: 5′-ACCTCACACC-3′) and *ArbPrim2* (11mer: 5′-ATGCAGAGTCC-3′) served as multiple binding arbitrary primers. PCR was performed with 80 pmol of each of the corresponding primers with the following cycle conditions: 40 cycles with 94°C for 30 s, 42°C for 60 s, and 72°C for 30 s. The PCR was repeated in the presence of ^{35}S-dATP (10 μCi, 1000 Ci per mmol; Amersham) using nested LTR primers (*LTR-K2* 5′-GTTCTTTCTCTAAGCTTTGT-CTCTGTG-3′ or *LTR-H2*: 5′-ACACAAAGCCTGTTTGGTGGTCT-CTTC-3′) in combination with the corresponding arbitrary primer. PCR products were then separated on a 5% nondenaturing polyacrylamide sequencing gel in 1 × TBE (90 mM Tris, 90 mM borate, 2 mM EDTA). Gels were fixed in 20% ethanol for 30 min, dried at 80°C and finally exposed to Kodak X-Omat AR films. Differentially expressed PCR products were excised from dried gels and gel slices were incubated in 400 μl H_2O for at least 12 h at 56°C. Eluted fragments were reamplified using identical primer sets and PCR conditions as described above.

Northern blot analysis Twenty micrograms of cytoplasmic RNA isolated from untreated and UVB-irradiated HaCaT cells were denatured by incubation in glyoxal (1 M, 1 h, 50°C) and electrophoretically separated (1 V per cm) in 1% agarose gels (10 mM Na_2HPO_4, pH 7.2). After transfer to a nylon membrane (Zetaprobe, BioRad, München, Germany) by vacuum blotting using 10 × SSC, hybridization was carried out in 0.5 M

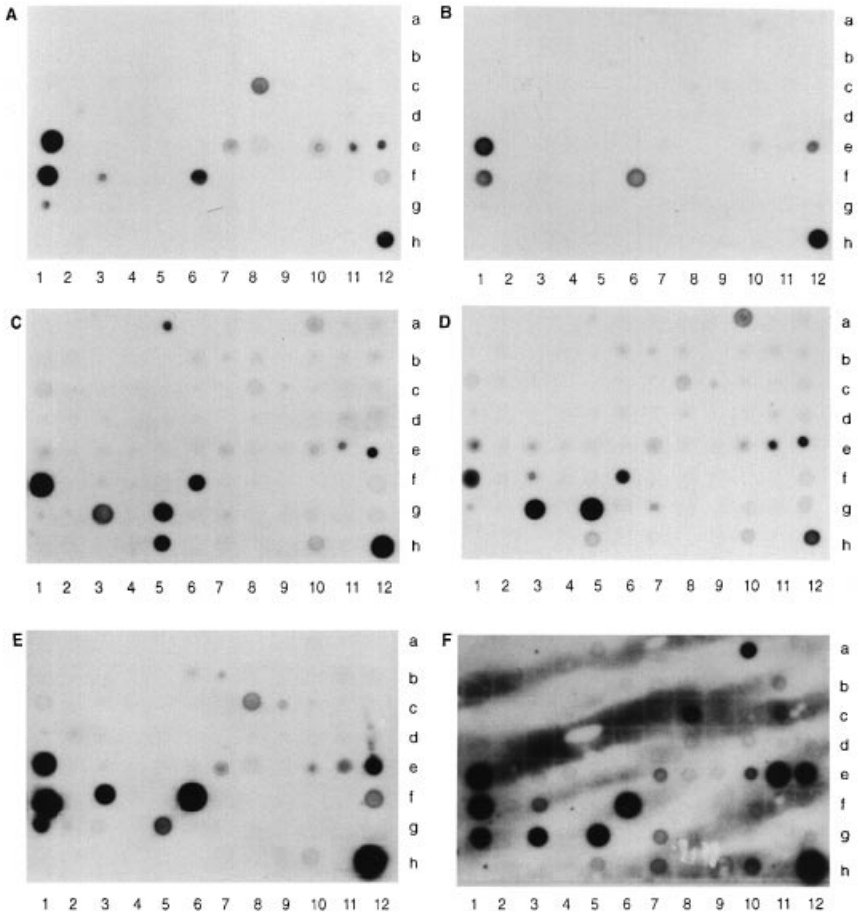

Figure 1. UVB-induced expression of HERV *pol* sequences in normal human primary epidermal keratinocytes and a keratinocyte cell line (HaCaT). Reverse dot blot hybridizations were performed with reverse transcribed poly(A)$^+$-RNA isolated from primary epidermal keratinocytes (NHEK) (*A*, *C*, *E*) and the keratinocyte cell line HaCaT (*B*, *D*, *F*). cDNA was amplified by PCR using degenerate oligonucleotide primers derived from a conserved region of retroviral *pol* genes. Radioactively labeled PCR products were subsequently hybridized to a representative number of defined *pol* fragments isolated and cloned from genomic DNA. The basis level expression of HERV *pol* sequences in untreated cells is shown in autoradiographs A and B. NHEK and HaCaT cells then were irradiated with 30 mJ UVB per cm^2 and further incubated for 6 h (*C*, *D*) and 24 h (*E*, *F*). Poly(A)$^+$-RNA was prepared, reverse transcribed and amplified as described above. The reverse dot blot hybridization clearly demonstrates transcriptional activation of additional endogenous *pol* sequences in UVB-irradiated cells compared to untreated controls.

Na_2HPO_4, pH 7.2, 7% SDS overnight at 65°C in the presence of denatured probe at 1×10^6 c.p.m. per ml. Membranes were then washed twice in 40 mM Na_2HPO_4, pH 7.2, 5% SDS for 30 min at 65°C and exposed to Kodak X-Omat AR films. For rehybridization membranes were washed twice in 0.1 × SSC, 0.5% SDS for 20 min at 95°C.

Cloning and sequencing of PCR products Reamplified PCR products were digested with the appropriate enzymes, purified, ligated into a *Sma*I- or *Hin*dIII/*Bam*HI-linearized pBluescript vector (Stratagene, Heidelberg, Germany) and transformed into *Escherichia coli* XL1-blue (Stratagene). DNA was extracted from different clones and sequenced with an Applied Biosystems sequencer (ABI 373A) according to the dsDNA dye terminator cycle sequencing protocol of Perkin Elmer.

Computer analyses Nucleotide sequences were compared with GenBank and EMBL data bases (release 110.0 and 56.0, respectively) using the FASTA program (Genetics Computer Group software).

Nucleotide sequence accession number The nucleotide sequence of clone H07 has been deposited in GenBank with accession number AF058295.

RESULTS

Basis level expression of endogenous retroviral sequences in human epidermal keratinocytes To investigate the expression of HERV in those cells of the human skin which are predominantly exposed to UV radiation *in vivo*, a spontaneously immortalized keratinocyte cell line (HaCaT) as well as primary epidermal keratinocytes (NHEK) were used as model systems. Expression was analyzed by RT–PCR of cellular poly(A)$^+$-RNA using degenerate primers derived from a highly conserved region within the retroviral *pol* gene (Shih *et al*, 1989; Donehower *et al*, 1990). Reamplified radioactively labeled *pol* products were hybridized in a reverse dot blot analysis to a representative number of defined HERV *pol* fragments isolated and cloned from human genomic DNA as previously described (Herrmann and Kalden, 1994). The results revealed a similar basis level expression pattern of HERV *pol* sequences in both primary (NHEK), and immortalized keratinocytes (HaCaT) (**Fig 1*A*, *B***). *Pol* transcripts expressed in untreated keratinocytes mainly belong to the ERV9, HERV-L, and HERV-H families as determined by database analyses of the known sequences of the dotted *pol* fragments (**Table I**, –UVB). Besides the five *pol* sequences expressed in both cell lines (**Fig 1**: 1e, 12e, 1f, 6f, 12h), untreated NHEK cells revealed a number of additional hybridization signals which appear in HaCaT cells only weakly (**Fig 1**: 10e, 11e) or, as described below, after UV treatment (**Fig 1**: 8c, 7e, 8e, 3f, 1g). This slightly different expression pattern of primary keratinocytes may reflect influences of culture conditions. For example, medium supplements such as epidermal growth factor (EGF), insulin, and hydrocortisone are essential for NHEK cell growth, and these substances may induce a response resembling a preactivated status in NHEK cells compared with untreated HaCaT cells.

Activation of HERV pol sequences in human keratinocytes by UVB irradiation The reverse dot blot hybridization analysis was repeated in at least three different experiments with NHEK and HaCaT cells after irradiation with various doses of UVA and UVB. Sublethal doses of the respective radiation were defined by Trypan blue exclusion tests (Lindl and Bauer, 1989) of irradiated cells. At the maximum doses of 20 J per cm^2 UVA and 30 mJ per cm^2 UVB 95% of the analyzed cells were found to be alive at 24 h post-irradiation. Cells were harvested 6 h and 24 h post-UV exposure. Irradiation of keratinocytes with low doses of UVB (10 mJ per cm^2) or even high doses of UVA (up to 20 J per cm^2) had little or no effect on HERV expression (data not shown). As shown in **Fig 1**, however, irradiation of these cells with 30 mJ per cm^2 UVB reproducibly led to transcriptional activation of a number of additional endogenous retroviral *pol* sequences (**Fig 1*C–F***) not

Table I. Expression patterns of HERV *pol* sequences in human epidermal keratinocytes as determined by reverse dot blot hybridization[a]

–UVB		+UVB	
Position	Identity	Position	Identity
8c[b]	HERV-E (4-1), 87.3% (M96062)	5a[bc]	HERV-K-T47D, 96.3% (*AF020092*)
1e[bc]	ERV9-related (MS associated) 89.1% (*A60201*)	10a[bc]	HERV-K-T47D (ERV-MLN), 91.7% (*U27242*)
8e[b]	HERV-K (HML6) clone 15H 81.9%;(*AF050507*)	7b[bc]	HERV-K, (RT244, MS brain), 91.1% (*S77583*)
10e[bc]	HERV-L, 88.1% (*AJ233673*)	11b[c]	human tigger1 element, 81.1% (*U49973*)
11e[bc]	ERV9 (pHE1), 84.8% (*M85205*, *X57147*)	1c[bc]	HERV *pol*, LE patient, 85.7% (*S66676*)
12e[bc]	ERV9 (RT18, MS brain), 90.5% (*S77576*)	8c[c]	HERV-E (4-1), 87.3% (*M96062*)
1f[bc]	HERV-H (Hp3), 86.9% (*S61068*)	9c[bc]	HERV-K, (RT244, MS brain), 90.0% (*S77583*)
3f[b]	ERV9-related (MS associated), 96.91% (*A57086*)	11c[c]	human tigger1 element, 82.6% (*U49973*)
6f[bc]	HERV-H (Hp3), 88.8% (S61068)	8d[c]	HERV-L, 82.0% (*X89211*)
1g[b]	human poly (ADP-ribose) *pol*, 90.9% (*M60436*)	10d[c]	ERV FRD (T47-D cells), 95.6% (*U27240*)
		3e[c]	HERV-L, 85.9% (*AJ233629*)
		3f[c]	ERV9-related (MS associated), 96.91% (*A57086*)
		1g[c]	human poly (ADP-ribose) *pol*, 97.6% (*M60436*)
		5g[b]	HERV-H (RGH2), 64.9% (*D11078*)

[a]The known sequences of dotted cloned *pol* fragments hybridizing with cDNA either from untreated (–UVB) and/or irradiated (+UVB) keratinocytes were compared with sequences present in the *GenEMBL* database. The grade of identity of each overlap is shown in percentage. Accession numbers are indicated in parentheses.
[b]HERV *pol* sequences expressed in normal human epidermal keratinocytes (NHEK).
[c]HERV *pol* sequences expressed in the keratinocyte cell line HaCaT.

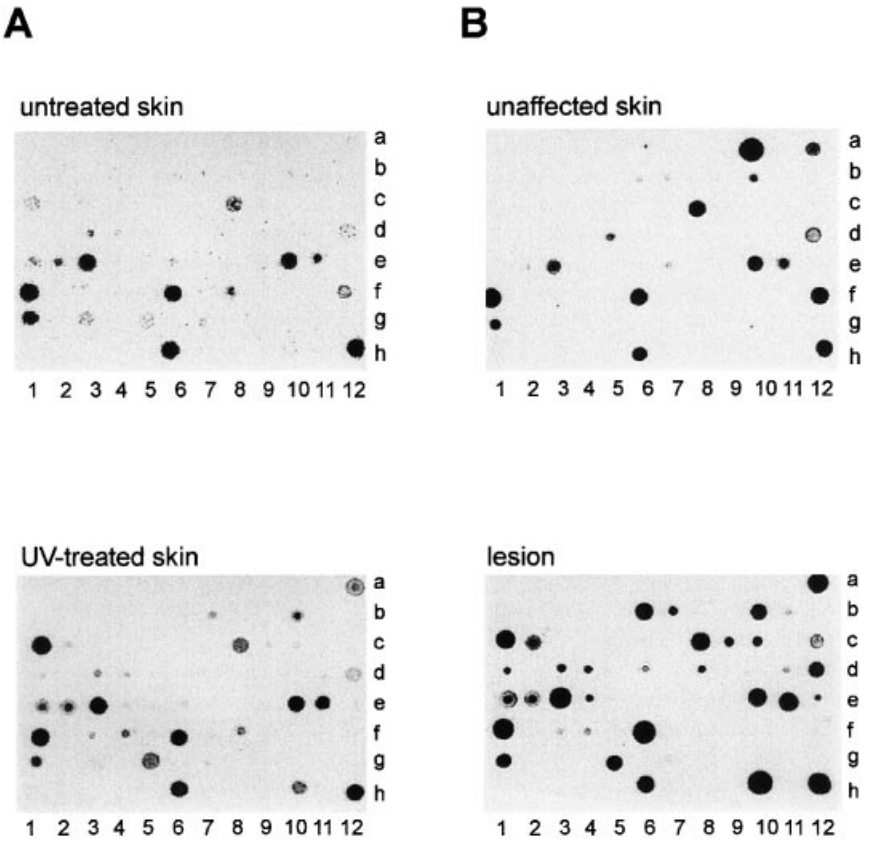

Figure 2. Activated HERV *pol* expression in skin biopsies of LE patients. HERV *pol* expression was analyzed by reverse dot blot hybridization with reverse transcribed poly(A)$^+$-RNA isolated from skin biopsies (4 mm in diameter) of different LE patients. Biopsies taken from UV-irradiated skin of a patient with systemic LE 3 wk after phototesting (*A*, UV-treated) were compared to an untreated control of the same patient (*A*, untreated). (*B*) Demonstrates the results for biopsies taken from skin lesions of a patient diagnosed with subacute cutaneous LE (*B*, lesion) compared with unaffected skin of the same patient (*B*, unaffected).

detected in the untreated controls (**Fig 1*A*, *B***). Expression patterns of UVB-treated primary keratinocytes and the keratinocyte cell line HaCaT were again similar. By means of database comparisons of the known dotted *pol* sequences, activated *pol* transcripts could be assigned to a variety of HERV elements, in particular to different members of the HERV-K, HERV-L and ERV9 families (**Table I**, +UVB). Some of the hybridizing dotted *pol* fragments (**Table I**: 7b, 1c, 9c) share high similarities with HERV transcripts found in patients with autoimmune diseases like systemic lupus erythematosus or multiple sclerosis (Kalden and Herrmann, 1993; Lefebvre *et al*, 1995).

Furthermore, the transcriptional activation of HERV *pol* sequences by UVB shows time-dependent alterations. Examination of expression patterns 6 h post-UV treatment revealed hybridization signals at this time point which vanish again 24 h post-irradiation (**Fig 1*C***: 5a, 10a, 3g, 5h). Other activated sequences were found to be expressed at both time points (**Fig 1*C***, ***E***: 5g, 10h) or to appear only 24 h after irradiation (**Fig 1*F***: 11b, 11c, 7h). Besides activating transcription, UV irradiation also seems to have inhibitory effects on the expression of certain HERV *pol* sequences. Some of the hybridization signals found in untreated NHEK cells (**Fig 1*A***: 8c, 1e, 3f) were no longer observed 6 h after irradiation (**Fig 1*C***).

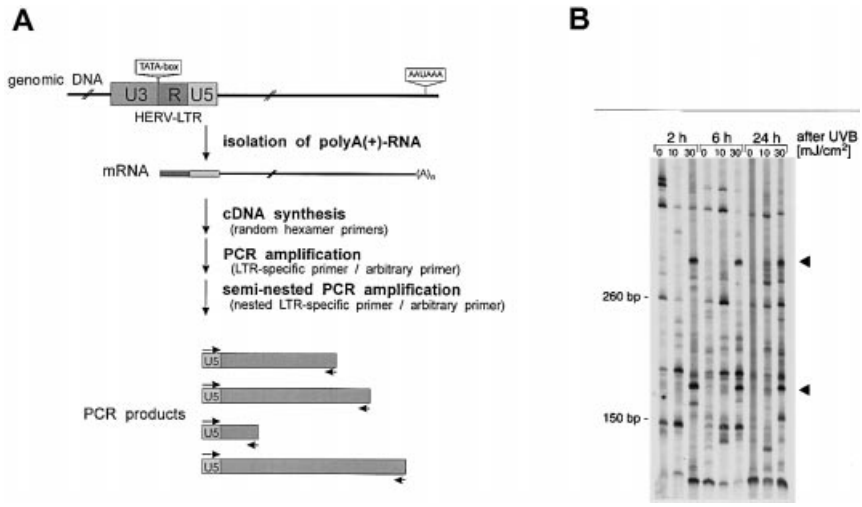

Figure 3. LTR-anchored differential display RT–PCR. (*A*) Schematic representation of LTR-specific DDRT–PCR To detect UV-induced HERV LTR-driven cellular transcripts, mRNA was extracted from human keratinocytes (HaCaT) before and after UVB irradiation. cDNA was synthesized by reverse transcription using random hexamer oligonucleotides. In a first round of PCR, cDNA was amplified using LTR-specific primers in combination with short arbitrary primers. In order to increase the proportion of LTR-containing cDNAs, a second PCR was performed with a nested LTR primer and the same arbitrary primer. LTR-specific primers were designed according to conserved sequences contained in the LTR U5 region of HERV-H and HERV-K. (*B*) Autoradiograph of a DDRT–PCR gel. Poly(A)$^+$-RNA was isolated from untreated HaCaT cells and from HaCaT cells irradiated with 10 and 30 mJ UVB per cm^2 (280–370 nm) and harvested 2 h, 6 h, and 24 h after UVB treatment. Synthesized cDNA was amplified by PCR as described in *Materials and Methods* using an HERV-H-specific LTR primer. Amplified products were analyzed on a 5% nondenaturing polyacrylamide gel. The *arrowhead* specifies the UVB-activated PCR product H07 which was analyzed in more detail.

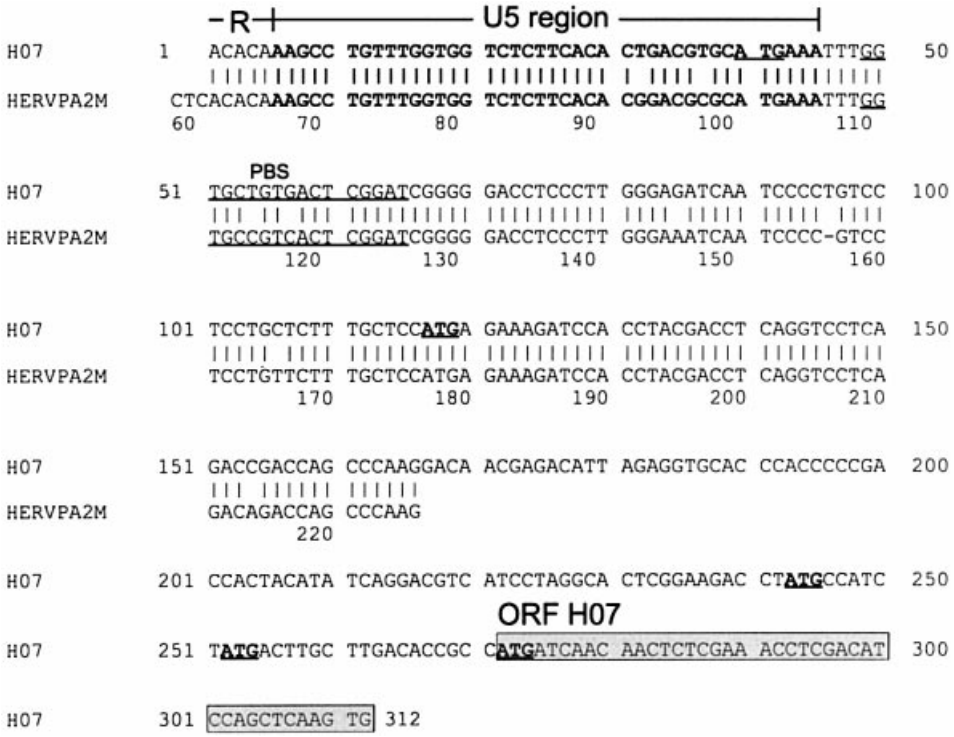

Figure 4. Sequence analysis of an UVB-activated LTR-driven cellular transcript. Database comparison of the 312 bp DDRT–PCR product H07 (accession number AF058295) obtained from UVB-treated HaCaT cells using HERV-H-specific LTR primers revealed 94.5% identity over 168 bp with an HERV-H 5′ sequence (HERVPA2M, accession number Z14310). No significant identity to the adjacent nonviral part of the sequence was found in the GenBank/EMBL database. Fragment H07 contains a number of potential open reading frames indicated by start codons (ATG). One of them, ORF-H07 starts at position 272 and exceeds beyond the cDNA fragment.

Induced expression of HERV pol sequences in skin biopsies of LE patients Using the reverse dot blot hybridization technique, expression of retroviral *pol* gene expression was analyzed in skin biopsies of LE patients who had been subjected to UV phototesting (Walchner *et al*, 1997) or exhibited spontaneous skin lesions (**Fig 2**). These samples were compared with unaffected or UV-untreated skin of the same patients. Both, biopsies from UV-irradiated areas (**Fig 2*A***, UV treated) or skin lesions (**Fig 2*B***, lesion) revealed enhanced expression of endogenous retroviral *pol* sequences in comparison with the respective control (**Fig 2*A***, untreated; **Fig 2*B***, unaffected). Activation patterns were found to be similar to UV-activated HERV *pol* expression in cultured keratinocytes (**Fig 1*C–F***). Activated sequences could again be assigned to the HERV families HERV-K (**Fig 2**: 7b, 1c, 2c, 9c), HERV-L (**Fig 2**: 3d, 4d, 8d, 2e), and ERV9 (**Fig 2**: 1e, 12e, 4f).

Detection of UVB-activated HERV LTR-driven expression of cellular genes by LTR-anchored DDRT–PCR Besides full length endogenous retroviral elements the human genome contains a large number of solitary LTR harboring all regulatory elements necessary for gene transcription. In fact, some of these LTR have been shown to be biologically active providing promoter/enhancer sequences or polyadenylation signals for the expression of cellular genes (Goodchild *et al*, 1992; Feuchter-Murthy *et al*,

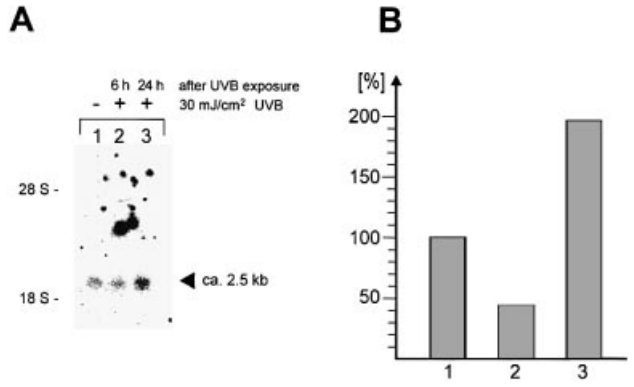

Figure 5. Detection of H07 transcripts by northern blot analysis. (*A*) Cytoplasmic RNA was isolated from untreated HaCaT cells (*lane 1*) and from HaCaT cells harvested 6 h (*lane 2*) and 24 h (*lane 3*) after irradiation with 30 mJ UVB per cm^2. The blot was hybridized with a 270 bp H07-specific fragment obtained by radioactive PCR using the oligonucleotides LTR-H3 (5′-GAAATTTGGTGCTGTGACT CGGA-TC-3′) and H07rev (5′-ACTTGAGCTGGATGTCGAGGTTTCG-3′) as primers. (*B*) Quantitative analysis of expression was performed with a phosphorimager (BAS 1000, Fuji) and the corresponding software (Tina 2.09G). The calculated differences normalized to hybridization with β-actin are given in percentage.

1993; Di Cristofano *et al*, 1995). Therefore, the effect of UVB radiation on transcriptional activation of HERV LTR-driven cellular genes was investigated. In order specifically to detect transcripts which are either driven by solitary endogenous retroviral LTR or are readthrough transcripts initiated from the 3′ LTR of an intact HERV element, we developed a modified version of the DDRT–PCR first described by Liang and Parde (1992) using stringent LTR-specific primers combined with a semi-nested PCR approach (Seifarth *et al*, 1999). After poly(A)$^+$-RNA isolation from untreated or irradiated HaCaT cells cDNA was synthesized. For PCR amplification, we chose primer sets consisting of LTR-specific and arbitrary oligonucleotides instead of using oligo(dT)-primers in combination with short arbitrary oligonucleotides (**Fig 3*A***). Based on the results obtained by reverse dot blot hybridization, LTR-specific primers were designed according to conserved sequences within the U5 LTR region of HERV-K (Ono *et al*, 1986; Leib-Mösch *et al*, 1993) and the R-U5 LTR region of HERV-H (Goodchild *et al*, 1993). In a sequential PCR, nested U5 LTR-specific primers were used in combination with the same arbitrary oligonucleotide in order to increase the sensitivity and specificity of LTR-bearing cDNA amplification. PCR amplified and radioactively labeled cDNAs of UVB-irradiated and untreated HaCaT cells were separated on high resolution polyacrylamide gels. **Figure 3**(***B***) shows an autoradiograph of the separated products obtained with a DDRT–PCR using HERV-H LTR-specific primers. Cells were treated with 10 mJ per cm^2 or 30 mJ per cm^2 UVB, and harvested at 2 h, 6 h, and 24 h after irradiation. For each lane a defined pattern of various bands in a size range of 150–500 bp was observed. Comparison of patterns before (**Fig 3*B***, lane 0 mJ per cm^2) and after irradiation (**Fig 3*B***, lanes 10 and 30 mJ per cm^2) revealed that some bands disappear whereas others arise after UVB treatment, corresponding to downregulated and upregulated mRNAs. A differential expression pattern was also obtained applying HERV-K specific primers (data not shown). In addition, as shown in the reverse dot blot hybridization experiments for endogenous *pol* transcripts, a time-dependent regulation of expression was also observed by DDRT–PCR. The pattern of differentially expressed cDNAs was demonstrated to be reproducible and specific depending on the LTR primer used for PCR.

Analysis of isolated UVB-induced cDNA fragments Focusing primarily on UV-activated expression, cDNA fragments which appeared consistently after UV treatment were excised and eluted from the gel (**Fig 3*B*,** *arrowheads*). DNA was reamplified applying the same primer sets and PCR conditions as before. Using this method, about 30 different cDNA fragments were cloned and sequenced. Database comparisons of the sequences obtained revealed that most of them have not yet been described. Some of the isolated PCR products showed considerable identity with LTR of known retrovirus-like elements such as HERV-K18 (Ono *et al*, 1986), HERV-H/RGH2 (Hirose *et al*, 1993), or HERV-H/cH7 (Mager, 1989) demonstrating the practicability of the modified DDRT–PCR approach. One of the LTR-bearing transcripts, the 312 bp cDNA fragment H07, showed 94.5% identity in a 168 bp overlap within the 5′ part of the HERV-H/PLA2 sequence (Feuchter-Murthy *et al*, 1993) including a stretch of 45 bp identity with the R-U5 LTR region and a 123 bp identity to the untranslated HERV-H leader sequence (**Fig 4**). This stretch is followed by 144 bp of a so far unknown cellular sequence. Preliminary analyses of potential open reading frames indicated by a start codon (**Fig 4**, ATG) and particularly of the one extending beyond the cDNA fragment (**Fig 4**; ORF-H07, start at position 272) did not reveal similarities to any known protein sequences.

Expression of H07 sequences in UVB-irradiated HaCaT cells In order to verify the H07-specific data obtained by DDRT–PCR, northern blot experiments were performed. HaCaT cells were irradiated with 30 mJ per cm^2 and harvested 6 h and 24 h post-treatment. Cytoplasmic RNA was isolated and analyzed in comparison to RNA extracted from untreated HaCaT cells by hybridization with an H07-specific probe which was generated by PCR using oligonucleotide primers LTR-H3 and H07rev designed according to the determined H07 sequence (**Fig 4**). As shown in **Fig 5*A***, the H07 fragment hybridized specifically to a single transcript of about 2.5 kb in length which is present in both RNA from untreated (**Fig 5*A*,** *lane 1*) and irradiated cells (*lanes 2* and *3*). Quantitative analysis based on hybridization with a human β-actin-specific probe, however, revealed differential expression of the H07 transcript. The calculated differences are shown in the corresponding histogram (**Fig 5*B***). It was found that transcription is first downregulated 6 h postirradiation (**Fig 5*B*,** *lane 2*) to less than half of the expression in untreated cells (**Fig 5*B*,** *lane 1*) and then upregulated 24 h after UVB treatment to about 2-fold of basis level expression (**Fig 5*B*,** *lane 3*).

DISCUSSION

Skin is the prominent target organ for natural UV radiation and therefore epidermal keratinocytes were chosen in order to investigate the influence of UVA and UVB on expression of HERV. Although it has been reported that UVA in a dose range of 10–20 J per cm^2 is able to induce transcription of cellular genes such as collagenase-I (Petersen *et al*, 1995) or heme oxygenase-1 (Keyse and Tyrrell, 1989) in cultured human skin fibroblasts, in this study UVA did not exert any noticeable effect on HERV transcription in epidermal keratinocytes (data not shown). We show for the first time, however, that UVB radiation in these cells causes transcriptional activation of a number of different HERV families. Most of the activated sequences are related to HERV elements which are biologically active in human tumor cells and tissues, such as HERV-K-T47D (Seifarth *et al*, 1995). Interestingly, some of the *pol* sequences hybridizing with UVB-activated HERV transcripts, i.e., ERV9 and HERV-K-related elements (see **Table I**), have been shown to be expressed in brains of multiple sclerosis patients (Lefebvre *et al*, 1995) or peripheral blood mononuclear cells of LE patients (Kalden and Herrmann, 1993). Recently, a multiple sclerosis-associated retrovirus was isolated from lepto-meningeal cells of a multiple sclerosis patient and shown to be closely related to ERV9 (Perron *et al*, 1997; Blond *et al*, 1999). In addition, analysis of HERV *pol* expression in skin biopsies of LE patients by reverse dot blot hybridization indicated activated HERV transcription in UV-provoked areas as well as in LE lesions. Analogous to the *in vitro* cell culture model, activated sequences could mainly be assigned to biologic active HERV members including those which have been described in particular in connec-

tion with autoimmune diseases. Finally, sequence analyses of individual activated *pol* transcripts isolated from hybridized dots revealed similarity as well as identity to the original dotted *pol* fragment (data not shown), suggesting that a broad spectrum of closely related HERV elements can be activated by UVB.

In addition to the induced transcription of HERV *pol* genes, we detected UVB-activated chimeric transcripts containing HERV LTR and cellular sequences by developing a LTR-anchored differential display reverse transcriptase–PCR approach. One of the isolated chimeric transcripts, the cDNA fragment H07, was shown to consist of a sequence identical to the 5′ end of an HERV-H element and a so far unknown cellular sequence. Northern blot hybridizations revealed a 2.5 kb transcript which was demonstrated to be differentially expressed in a time-dependent manner in UVB-irradiated HaCaT cells. Normalization to β-actin expression, which is known to be not influenced by UVB (Imokawa *et al*, 1992; Rosen *et al*, 1992), demonstrated a 2-fold increase in the H07 transcript 24 h after irradiation. Similar magnitudes of activation were recently demonstrated for a number of cellular sequences which have been isolated from UVB-irradiated rat keratinocytes by cDNA subtraction (Rosen *et al*, 1995) and from human keratinocytes using DD-PCR (Abts *et al*, 1997). The primary structure of the H07 transcript indicates that it might be the result of a splicing event between an HERV-H element and a cellular sequence. The presence and even biologic relevance of such chimeric transcripts especially with participation of HERV-H has been demonstrated in several examples (Feuchter-Murthy *et al*, 1993; Di Cristofano *et al*, 1995).

Various studies in the last few years have addressed the mechanisms which are responsible for UV-dependent gene activation (reviewed in Bender *et al*, 1997). First, membrane components, including growth factor receptors for EGF and insulin, seem to act as sensors for UV irradiation mediating a kind of immediate early reaction in irradiated cells by autophosphorylation. Following receptor phosphorylation, soluble membrane associated factors such as tyrosine kinases of the *src*-family and Ha-*ras* small GTP-binding protein have been shown to be activated by UV exposure. The rapid response (within 30 min) is mainly due to activation of various transcription factors via phosphorylation through different UV-activated protein kinases. Immediate response genes include those coding for the transcription factors Fos and Jun.

Different cell types, including keratinocytes, have been found to produce various cytokines such as tumor necrosis factor-α, basic fibroblast growth factor, granulocyte-macrophage colony- stimulating factor, interleukin (IL)-1α and IL-6 in response to UV radiation (Funk and Maibach, 1994). It is quite likely that the late response to UV irradiation (in the range from 4 h to 36 h) may be mediated through an extracellular loop involving the action of growth factors and interleukins. This hypothesis is further supported by reverse dot blot hybridization experiments demonstrating activation of HERV *pol* sequences in HaCaT cells after incubation in conditioned culture medium obtained from UVB-irradiated cells (data not shown). HERV *pol* expression patterns of UVB-treated cells 24 h after irradiation and of cells cultured with conditioned medium for the same time turned out to be nearly identical. Some additional *pol* transcripts, however, which appeared only in UVB-irradiated HaCaT cells indicate involvement of others than soluble factors contributing to HERV activation. In order to obtain more information which of the mentioned cytokines may be relevant for UVB activation of HERV transcription in human keratinocytes, expression of the LTR-initiated transcript H07 was analyzed in response to EGF, tumor necrosis factor-α, IL-1α, IL-6, and a combination of all (data not shown). The results revealed only a slight increase in H07 mRNA concentration (25–40%) after treatment with tumor necrosis factor-α or IL-1α, and even a decrease when cells were incubated with the cytokine mixture. This suggests a more complex regulation of UVB-activated HERV transcription, which will be subject of further investigations.

Besides influencing cellular transcription, UV radiation is further known to be one of the environmental factors able to induce programmed cell death (apoptosis) in various cells *in vitro* and *in vivo*, including human keratinocytes (Gunn *et al*, 1983; Schwarz *et al*, 1995). Significantly, cultivated HaCaT cells were shown to undergo apoptosis after exposure to the UV treatment used in the present study (Hagenhofer *et al*, 1998). UVB-irradiated human keratinocytes destined for cell death undergo morphologic changes characterized by the formation of apoptotic blebs at their cell surface (Casciola-Rosen *et al*, 1994). Analysis of these blebs revealed that they contained intracellular material such as nucleosomal DNA and small ribonucleoproteins, well documented as autoantigens in LE (Kotzin, 1996). Particularly in autoimmune diseases such as LE, Sjögren's syndrome (Krieg *et al*, 1992), or multiple sclerosis, endogenous retroviruses are discussed as potential etiologic agents or at least as a source of autoantigens detected by cross-reacting antibodies found in the sera of autoimmune patients (Talal *et al*, 1990a,b). Thus, the UV-induced transcriptional activation of HERV sequences demonstrated here combined with UV-induced specific apoptotic processes may result in the inappropriate presentation of HERV-encoded proteins and other cellular autoantigens to the immune system finally leading to autoantibody production.

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