# Late Cornified Envelope Family in Differentiating Epithelia—Response to Calcium and Ultraviolet Irradiation

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**The late cornified envelope (LCE) gene cluster within the epidermal differentiation complex on human chromosome one (mouse chromosome three) contains multiple conserved genes encoding stratum-corneum proteins. Within the LCE cluster, genes form ''groups'' based on chromosomal position and protein homology. We link a recently accepted nomenclature for the LCE cluster (formerly XP5, small proline-rich-like, late-envelope protein genes) to gene structure, groupings, and chromosomal organization, and carry out a pan-cluster quantitative expression analysis in a variety of tissues and environmental conditions. This analysis shows that (i) the cluster organizes into two ''skin'' expressing groups and a third group with low-level, tissue-specific expression patterns in all barrier-forming epithelia tested, including internal epithelia; (ii) LCE genes respond ''group-wise'' to environmental stimuli such as calcium levels and ultraviolet (UV) light, highlighting the functional significance of groups; (iii) in response to UV stimulation there is massive upregulation of a single, normally quiescent, non-skin LCE gene; and (iv) heterogeneity occurs between individuals with one individual lacking expression of an LCE skin gene without overt skin disease, suggesting LCE genes affect subtle attributes of skin function. This quantitative and pan-cluster expression analysis suggests that LCE groups have distinct functions and that within groups regulatory diversification permits specific responsiveness to environmental challenge.**

Key words: epidermal differentiation complex/LCE/skin barrier/ultraviolet
J Invest Dermatol 124:1062–1070, 2005

The epidermal differentiation complex (EDC) at 1q21 (human) and chromosome 3F1 (mouse) is enriched for genes associated with epidermal terminal differentiation (Backendorf and Hohl, 1992; Engelkamp *et al*, 1993; Mischke *et al*, 1996). As well as encoding single-terminal differentiation genes the EDC also contains "clusters" of related genes, e.g., S100 genes, small proline-rich region (SPRR) genes, and a recently identified gene cluster, the late cornified envelope (LCE) genes (previously XP5, EIG, small proline-rich-like, late envelope protein (LEP) genes) (Zhao and Elder, 1997; Marshall *et al*, 2001; Wang *et al*, 2001) (Fig 1).

The clustering of epidermal differentiation genes within the EDC suggests evolution via gene amplification, then diversification. Gene amplification within the S100 clusters has resulted in clear diversification of protein function (reviewed in Heizmann *et al*, 2002; Eckert *et al*, 2004). Diversification of function has not been formally demonstrated for SPRR proteins, which are protein cross-linking components of the keratinocyte-cornified envelope (Steinert *et al*, 1998a). But based on biochemical evidence (Steinert *et al*, 1998b), differential expression, and association of an SPRR isoform with a particular cornified envelope morphology (Cabral *et al*, 2001a), it has been proposed that isoforms have distinct functions related to modification of cornified envelope properties at different anatomical sites or in response to environmental stresses, such as ultraviolet (UV) irradiation (Kartasova and van de Putte, 1988; Hohl *et al*, 1995; Yaar *et al*, 1995; Kartasova *et al*, 1996; Song *et al*, 1999; Cabral *et al*, 2001a; Marshall *et al*, 2001; Wang *et al*, 2001; Patel *et al*, 2003).

An alternative interpretation of differential expression analyses is that closely related isoforms, although differentially expressed, have similar function (Cabral *et al*, 2001b). Within the SPRR cluster are subclusters or groups of closely related genes. Group 2 SPRR genes are very highly conserved within their coding sequences but show divergent, gene-specific expression patterns (Cabral *et al*, 2001b). This implies that evolution of promoter or regulatory diversity has been more important than diversification of SPRR protein sequences and that protein dosage is important for function (Gibbs *et al*, 1993; Cabral *et al*, 2001b). Hence, highly conserved groups within the SPRR cluster could constitute an "extended promoter," permitting far greater sensitivity to cellular and environmental stimuli than is possible to achieve with a single or few genes.

The human LCE cluster is very similar to the SPRR cluster in that (1) it encodes proteins related to SPRR proteins

Abbreviations: EDC, epidermal differentiation complex; LCE, late cornified envelope; LEP, late envelope proteins; SPRL, small proline-rich like; SPRR, small proline-rich region; SPRRL, small proline-rich region like; UV, ultraviolet

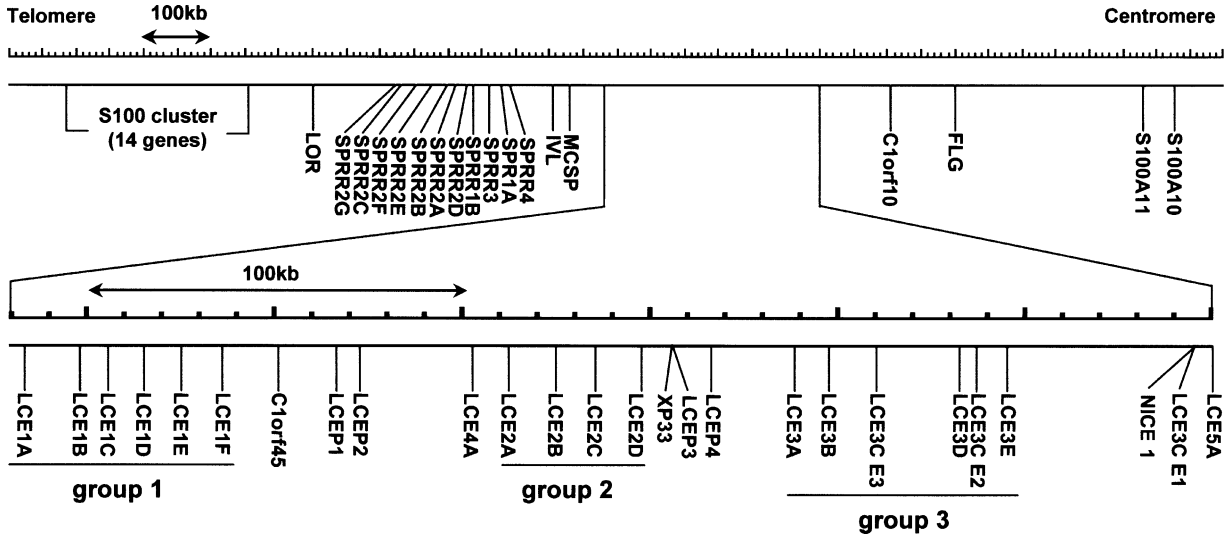


**Figure 1**
**Location of late cornified envelope (LCE) genes within the human epidermal differentiation complex (EDC).** Inset shows the LCE genes that cluster into three groups. E1, E2, and E3 correspond to the three exons of LCE3C. The terminal 51 nucleotides of LCE3C exon one (E1) correspond to the complete exon 1 of NICE-1. LCEP3 is located in the last bases of exon 2 and within intron 2 of XP33.

(Zhao and Elder 1997; Wang *et al*, 2001); (2) the proteins are also cornified envelope precursors that have protein cross-linking functions similar to SPRR (Marshall *et al*, 2001; Steinert *et al*, 2003); (3) the LCE cluster contains "groups" encoding conserved proteins; and (4) there is differential expression between group members (Marshall *et al*, 2001), although comparative expression within groups has not been investigated.

The large number of LCE genes and their amino acid sequence similarity begs the question "Why are there so many genes?" Theories related to the multiplicity of SPRR genes are probably relevant to LCE genes. A prerequisite for distinguishing between theories is quantitative data on expression levels across the LCE cluster and within LCE groups.

It has been difficult to compare analyses of LCE gene expression between laboratories (e.g., Zhao and Elder, 1997; Marshall *et al*, 2001; Wang *et al*, 2001; Jonker *et al*, 2004) because of the multiplicity of names associated with these genes. Therefore, the authors recently proposed a new nomenclature for the human genes and one aim of the work is to relate this nomenclature to genomic organization, amino acid sequence, and link the new nomenclature to previous nomenclatures. A second aim is to carry out a simultaneous pan-group quantitative expression analysis in a range of tissues and environmental conditions so that expression can be linked to the new nomenclature, and set a baseline for comparison of LCE expression under different clinical conditions. Such a pan-group analysis is a prerequisite for distinguishing between theories about cluster evolution and function.

## Results

**Nomenclature, genomic organization, and protein homology** The forerunner for this group of genes was XP5, a gene expressed in skin, including psoriatic skin (Zhao and Elder, 1997). Subsequently, further murine genes (small proline-rich region-like (SPRRL); Wang *et al*, 2001) and human genes (LEP; Marshall *et al*, 2001) were characterized. As the proteins encoded by these genes are precursors of the cornified envelope of the stratum corneum, and are not detected until relatively late during fetal assembly of the skin cornified envelope a new name, late cornified envelope or LCE, has recently been agreed and accepted by the HUGO Gene Nomenclature Committee (Table I).

The genomic organization of the LCE genes at 1q21 is demonstrated in Fig 1. The cluster stretches over 320 kb of the 2.5 Mb EDC. Based on genomic organization and predicted amino acid sequence LCE genes organize into three linked subclusters. As the genomic subclusters relate to conserved amino acid sequence and expression (Marshall *et al*, 2001), the gene names reflect these groups (Table I, Fig 1). In addition to the three groups there are fourth and fifth genes, LCE4A and LCE5A.

The predicted amino acid sequences fall into structure-based groups that correspond to genomic organization (Fig 2).

Four pseudogenes associate with the LCE cluster (Fig 1). Pseudogene 2 (LCEP2) is homologous to LCE5A and contains a cluster of stop codons internally. Although a pseudogene in most patients, expression has been detected in one individual, suggesting population heterogeneity. Interestingly, LCEP3, the least intact of the pseudogenes, is located within intron 2 of XP33, an unrelated skin-specific gene (Zhao and Elder, 1997, Fig 1).

**Gene structure and links with NICE-1** The intron/exon structures of LCE genes were determined by 5′ RACE and DNA sequencing (Fig 3), with the single exception of LCE3B, a gene whose messenger RNA (mRNA) is very rare and whose expression was verified by sequencing complementary DNA (cDNA) containing the second exon, with the first exon structure being based on homology prediction. Most of the genes conform to a two-exon structure

**Table I. Revised nomenclature for the LCE (formally XP5, SPRL, LEP) gene family**

| Chromosome position ( × 1000) | HGNC-approved nomenclature | Name | Aliases | RefSeq | Ref. |
|---|---|---|---|---|---|
| 149664 | MCSP | Mitochondrial capsule selenoprotein | | NM_030663 | 1 |
| 149629 | C1orf44 | Chromosome 1 open reading frame 44 | | | |
| 149613 | LCE1A | Late cornified envelope 1A | LEP1 | NM_178348 | 2 |
| 149598 | LCE1B | Late cornified envelope 1B | SPRL2A, LEP2 | NM_178349 | 3, 2 |
| 149591 | LCE1C | Late cornified envelope 1C | LEP3 | NM_178351 | 2 |
| 149583 | LCE1D | Late cornified envelope 1D | LEP4 | NM_178352 | 2 |
| 149572 | LCE1E | Late cornified envelope 1E | LEP5 | NM_178353 | 2 |
| 149562 | LCE1F | Late cornified envelope 1F | LEP6 | NM_178354 | 2 |
| 149546 | C1orf45 | Chromosome 1 open reading frame 45 | | | |
| 149530 | LCEP1 | Late cornified envelope pseudogene 1 | | | |
| 149523 | LCEP2 | Late cornified envelope pseudogene 2 | LEP7 | | 2 |
| 149494 | LCE4A | Late cornified envelope 4A | SPRL4A, LEP8 | NM_178356 | 3, 2 |
| 149484 | LCE2A | Late cornified envelope 2A | LEP9 | NM_178428 | 2 |
| 149472 | LCE2B | Late cornified envelope 2B | XP5, LEP10, SPRL1B | NM_014357 | 4, 2, 3 |
| 149462 | LCE2C | Late cornified envelope 2C | LEP11 | NM_178429 | 2 |
| 149450 | LCE2D | Late cornified envelope 2D | LEP12, SPRL1A | NM_178430 | 2, 3 |
| 149442 | LCEP3 | Late cornified envelope pseudogene 3 | | | |
| 149441 | C1orf46 | Chromosome 1 open reading frame 46 | XP33 | AF005082 | 4 |
| 149430 | LCEP4 | Late cornified envelope pseudogene 4 | | | |
| 149408 | LCE3A | Late cornified envelope 3A | LEP13 | NM_178431 | 2 |
| 149399 | LCE3B | Late cornified envelope 3B | LEP14 | NM_178433 | 2 |
| 149386 | LCE3C | Late cornified envelope 3C | SPRL3A, LEP15 | NM_178434 | 3, 2 |
| 149365 | LCE3D | Late cornified envelope 3D | SPRL6A, LEP16 | NM_032563 | 3, 2 |
| 149351 | LCE3E | Late cornified envelope 3E | LEP17 | NM_178435 | 2 |
| 149300 | C1orf42 | Chromosome 1 open reading frame 42 | NICE1 | NM_019060 | 5 |
| 149297 | LCE5A | Late cornified envelope 5A | SPRL5A, LEP18 | NM_178438 | 3, 2 |
| 149195 | C1orf10 | Chromosome 1 open reading frame 10 | | NM_016190 | 6 |

Flanking genes (e.g., MCSP, C1orf10) are shown to provide orientation (see Fig 1). Listing is from telomere to centromere.
1. Aho *et al* (1996); 2. Marshall *et al* (2001); 3. Wang *et al* (2001); 4. Zhao and Elder (1997); 5. Marenholz *et al* (2001); 6. Xu *et al* (2000).
HGNC, HUGO Gene Nomenclature Committee; LEP, late envelope proteins; SPRL, small proline-rich like.

(Fig 3, gray), with a single intron within the 5′ untranslated region (UTR) splitting the 5′ UTR into an $\sim$ 50 bp first exon and an $\sim$ 20 bp UTR region upstream of the coding sequence in the second exon. This structure occurs in involucrin, loricrin, most of the SPRR family, and NICE-1 (Eckert and Green, 1986; Yoneda *et al*, 1992; Gibbs *et al*, 1993; Fischer *et al*, 1999; Cabral *et al*, 2001b; Marenholz *et al*, 2001), suggesting, along with sequence conservation, divergence from a primordial gene (Backendorf and Hohl, 1992; Gibbs *et al*, 1993).

The exceptions to the structure are (1) LCE1B, 3D, and 3E (Fig 3 stippled) with a single exon, suggesting evolution by a splice site mutation; (2) LCE4A and 5A (Fig 3, black) with extended first exons but conserved second exons, suggesting duplication of these two related genes (Fig 2); (3) an LCE1E minor, alternatively spliced transcript containing a middle untranslated exon between the first and last exons (data not shown—expressed sequence tag data suggest at least one further splice variant that was not detected in this study); and (4) LCE3C (Fig 3, white) with an extended three-exon structure.

The three exons of LCE3C were identified by 5′ RACE using oesophagus mRNA and are spread over approximately 90 kb of DNA with additional LCE family members within the very large introns (Figs 1 and 3). Despite the unusual structure of LCE3C the third exon, containing the coding sequence is conserved with a short, 23 bp 5′ UTR, typical of this group of EDC genes. Interestingly, exon one of LCE3C is shared with neighboring NICE-1, suggesting common regulation and a shared promoter (Fig 1). NICE-1 encodes a 99 amino acid protein rich in glutamine, serine, and cysteine that is not expressed in skin but is expressed

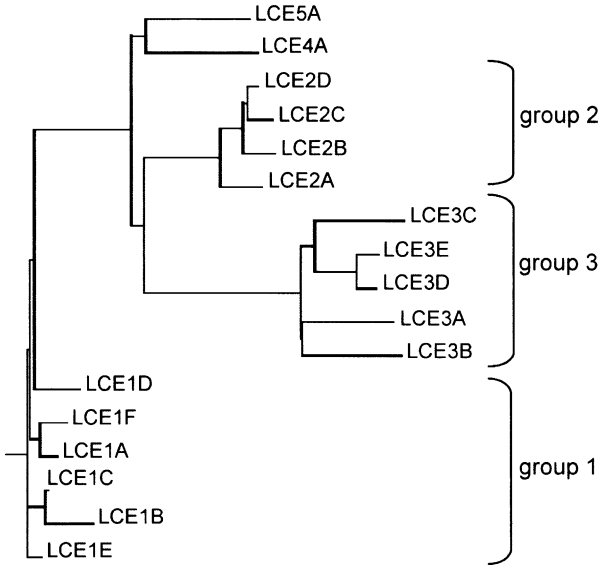


**Figure 2**
**Grouping of late cornified envelope (LCE) proteins based on primary sequence similarity.** PHYLIP rooted tree phenogram (Felsenstein, J. 1993. PHYLIP, Phylogeny Inference Package, version 3.5c) is based on predicted coding sequences. Groups correspond to chromosomal clusters except for LCE4A and LCE5A (Fig 1).

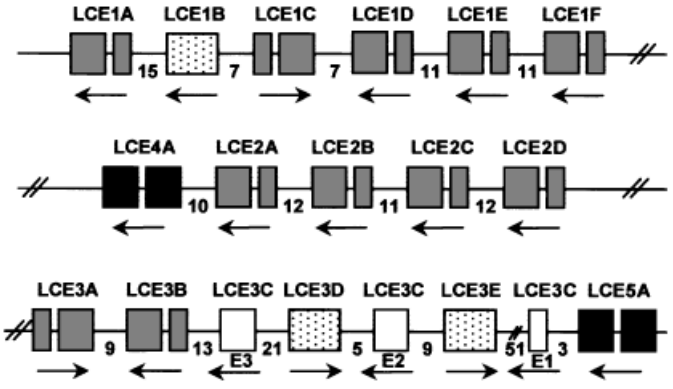


**Figure 3**
**Structure of late cornified envelope (LCE) genes.** Boxes show exon–intron structures and arrows indicate the direction of transcription. For clarity, the exons of LCE3C are individually labeled (E1–3). Numbers represent the distance between genes in kilobases. Black, gray, white, and stippled represent the four LCE gene structures described in the text. Intron/exon structure was established by 5′ RACE except for the structure of exon one of LCE3B, a very rare transcript, which was inferred from homology with other LCE group 3 genes.

in differentiating cultured keratinocytes (Marenholz *et al*, 2001), suggesting that *in vivo* it could express in internal stratum-corneum-forming epithelia. EST data show expression of NICE-1 in the heart and squamous cell carcinoma. NICE-1 and LCE proteins show very little similarity except at the extreme N-terminus, implying that NICE-1 has only a distant relationship with the LCE gene family (Marenholz *et al*, 2001). The significance of the shared first exon and promoter is not understood. LCE3C expression is not detected with NICE-1 in the heart.

**Quantitative expression analysis** LCE gene expression could not be detected in the cervix, rectum, lung, colon, or placenta using conventional, sensitive PCR. In contrast, LCE gene expression was readily detected in adult trunk skin, adult arm skin, fetal skin, penal skin, vulva, esophagus, and tongue (data not shown). Variable expression of some subtypes is detected in the heart (LCE5A) and fibroblasts (LCE1F).

Because levels of LCE expression may be important for function, quantitative analysis via real-time PCR was performed. In addition, this approach lends itself, theoretically, to simultaneous analysis of the entire LCE group. But in practice, the close conservation of LCE1D and E meant that these two genes had to be analyzed together. The specificity and quantitative authenticity of data were checked for every primer pair in every tissue and condition, as described in Materials and Methods (Table S1 and Fig S1).

Real-time analysis revealed expression levels for LCE genes across five orders of magnitude (Fig 4). The conclusions are: (i) groups 1 and 2 are the dominant "skin" expressing groups, being downregulated or undetectable in internal epithelia; (ii) LCE1C is the dominant skin gene, with LCE2A and LCE2B (formally XP5) prominent group 2 skin genes (Fig 4*A*); (iii) internal stratified epithelia such as tongue and esophagus do not express any group 1 or two genes, whereas vulva seems intermediate between external and internal epithelia, retaining low levels of group 1 and 2 expressions (Fig 4*B*); (iv) although non-quantitative expression analysis marks murine group 3 genes as being hallmarks of internal epithelia, quantitative analysis shows that the human group 3 genes are unexpressed or expressed variably at low-to-intermediate levels in internal and external epithelia, with expression profiles changing markedly between tissue types. Hence, human group 3 genes provide a "signature" for different types of differentiating epithelia (see Fig 4*B,C*); and (iv) LCE4A and LCE5A expressions were barely detected in the tissues surveyed.

A concern with expression analysis using human tissue RNA is interindividual expression heterogeneity as human tissue samples are, of necessity, from different individuals. Therefore, LCE group expression analysis was performed on biopsies from four human subjects at a single anatomical site (upper inner arm skin, non-sun exposed). The analysis (Fig 5) showed conserved profiles between individuals with conservation of the group 1 and 2 skin expression profile and reduced group 3, 4, and 5 expressions. But specific differences were detected between individuals—individual four lacks LCE1D/E expression almost entirely and LCE2B is reduced in individual two. This result suggests that there may be population heterogeneity in LCE expression that could translate to functional consequences. The almost total lack of LCE1D/E in one individual with no major skin defect suggests that functional consequences of loss of expression of a single gene would be minor.

**Environmental response—response to calcium** The most widely held theory for multiplicity of SPRR/LCE genes proposes differential responsiveness to environmental stimuli. Cultured keratinocytes respond to extracellular calcium by differentiating, monitored by induction of "markers" such as differentiation-specific keratins, then cornified envelope precursors such as involucrin, loricrin then filaggrin (Hennings *et al*, 1980). In addition to inducing keratinocyte

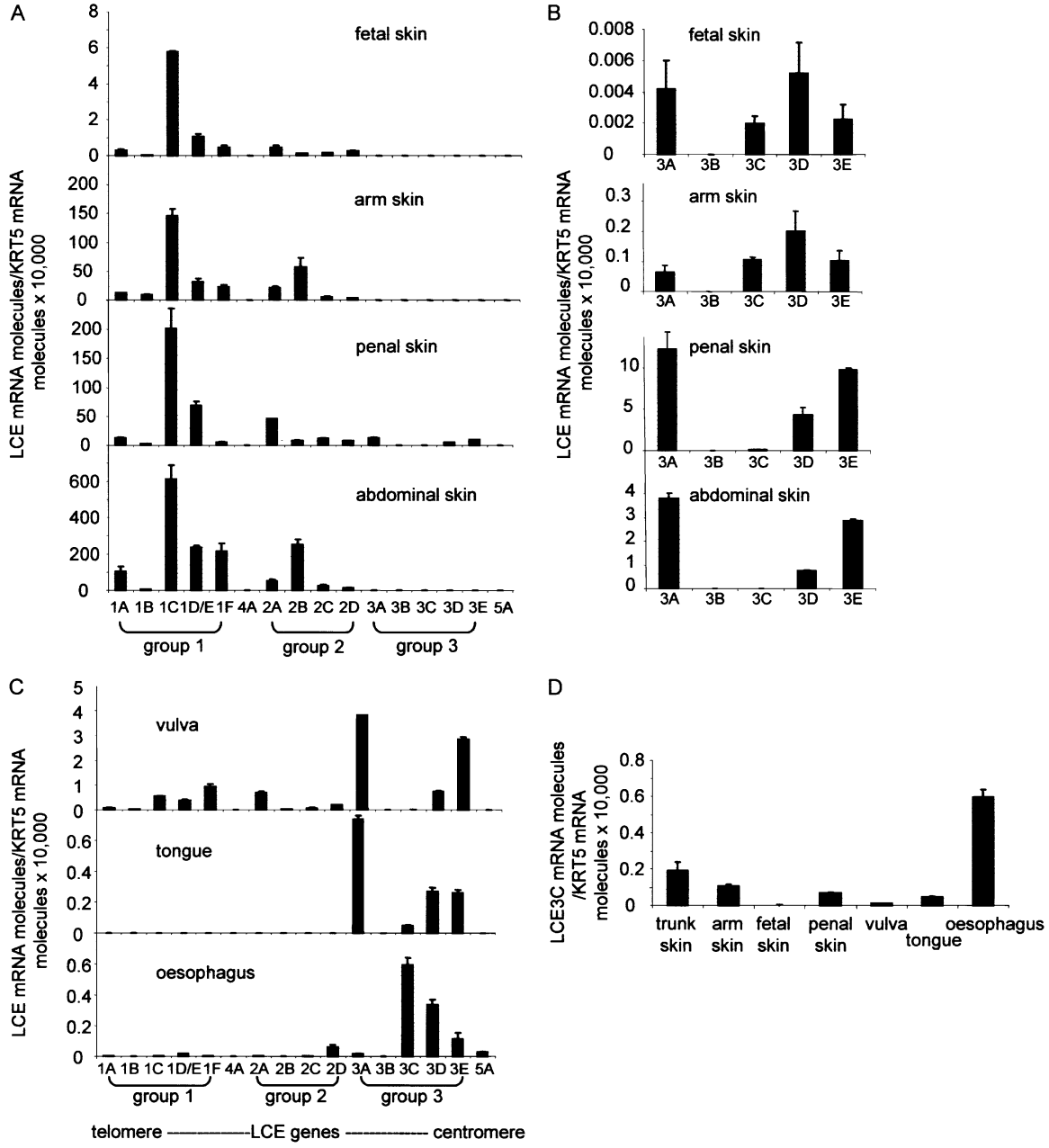


**Figure 4**
**Late cornified envelope (LCE) expression profiles in stratified, cornifying tissues.** (*A*) Skin expression profiles of LCE genes showing prominent group 1 and 2 expression but very low levels of group 3 expression. Note that fetal skin levels are two orders of magnitude lower that adult skin but still show a typical "skin"-type expression profile. (*B*) The scale of graphs from (*A*) has been reduced to show the very low, but reproducible, levels of group 3 LCE gene expression in skin. Note that group 3 gene profiles vary between skin types. Fetal skin, which was from a commercial source with unspecified body location, is again several orders of magnitude below adult skins but is closest to the arm skin profile. (*C*) Expression profiles of internal mucosal stratifying epithelia. Expressions of group 1 and 2 genes are only detected in vulva where they are several orders of magnitude lower than in the skin. There is no skin expression of group 1 and 2 genes in tongue and esophagus. (*D*) Comparison of a group 3 gene, LCE3C, in a range of tissues to illustrate how group 3 genes vary between tissue type and expression profiles is diagnostic of tissues. Genes are grouped in chromosomal position from telomere to centromere (compare Fig 1). *Error bars* ± SEM.

differentiation *per se*, specific keratinocyte genes are "calcium inducible" or have been reported to have "calcium response elements."

LCE expression is differentiation-dependent (Marshall *et al*, 2001); so to monitor calcium induction primary human keratinocytes were grown to confluence to induce differentiation (Li *et al*, 2001). Under these conditions, cultured keratinocytes show a "skin"-type LCE expression profile except that a group 3 gene, LCE 3E, is expressed (Fig 6). Raising extracellular calcium to 1.2 mM for 48 h results in specific upregulation of group 2 genes only (Fig 6). Hence, LCE genes show a group-specific sensitivity to extracellular calcium levels. This result does not rule out the possibility that other LCE genes are responsive to calcium since under the conditions of the experiment they may have been fully induced, or they may require longer exposure to high calcium for further induction. This result does show, however, that a single LCE group can respond group-wise to a

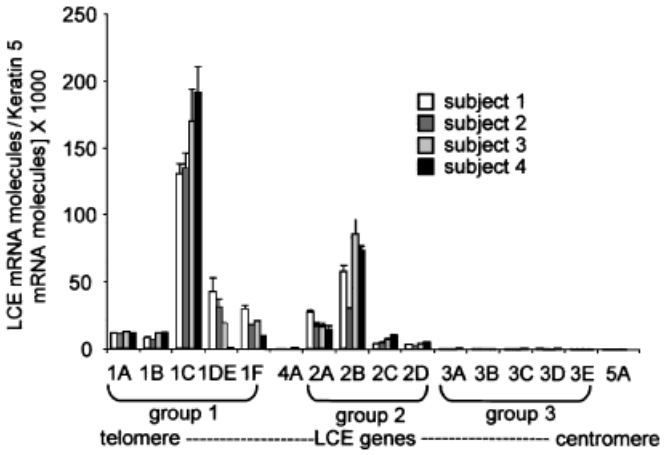


**Figure 5**
**Heterogeneity in late cornified envelope (LCE) expression.** Site-matched LCE real-time expression profiles from four age-matched, Caucasian volunteers (skin types 2–3) from identical sites (upper arm) show similar group 1 and 2 skin-type expression profiles with reduced group 3 expression; however, differences in LCE1D/E and LCE2B genes are detected. LCE expression is normalized to keratin 5 expression. *Error bars* ± SEM.

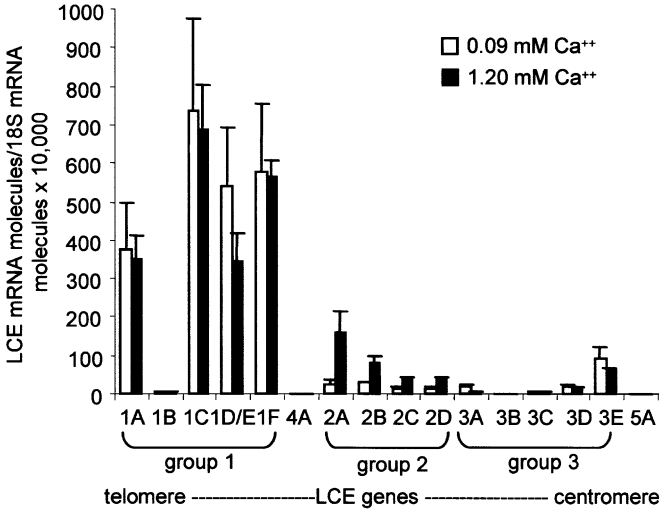


**Figure 6**
**Calcium responsiveness of late cornified envelope (LCE) genes.** Real-time expression profiles are from differentiated keratinocytes grown in low (0.09 mM) or high (1.5 mM calcium) for 48 h and show that group 2 genes alone are calcium-responsive. In cultured cells, LCE expression is normalized to 18S RNA levels. *Error bars* ± SEM.

changed environmental condition and that group 2 genes respond distinctly to calcium levels.

**Environmental response—response to UV light** Stratum corneum provides the primary barrier against UV irradiation and SPRR genes are selectively upregulated in response to UV. The response of LCE genes to UVB was analyzed in cultured primary human keratinocytes using a dosage (7.5 mJ per $cm^2$) that we (data not shown) and others (Li *et al*, 2001) show does not produce significant cell death over the time course of the experiment. Because previous investigators have shown cornified envelope changes to occur late (24 h) after irradiation (Li *et al*, 2001), with SPRR4 induced up to 48 h after irradiation (Cabral *et al*, 2001a), the time interval 24–48 h was chosen to monitor change (Fig 6). Keratinocytes were grown to confluence in low-calcium media before UV irradiation to induce differentiation (Li *et al*, 2001). Calcium-induced differentiation was avoided to permit discrimination between UV and calcium effects on expression.

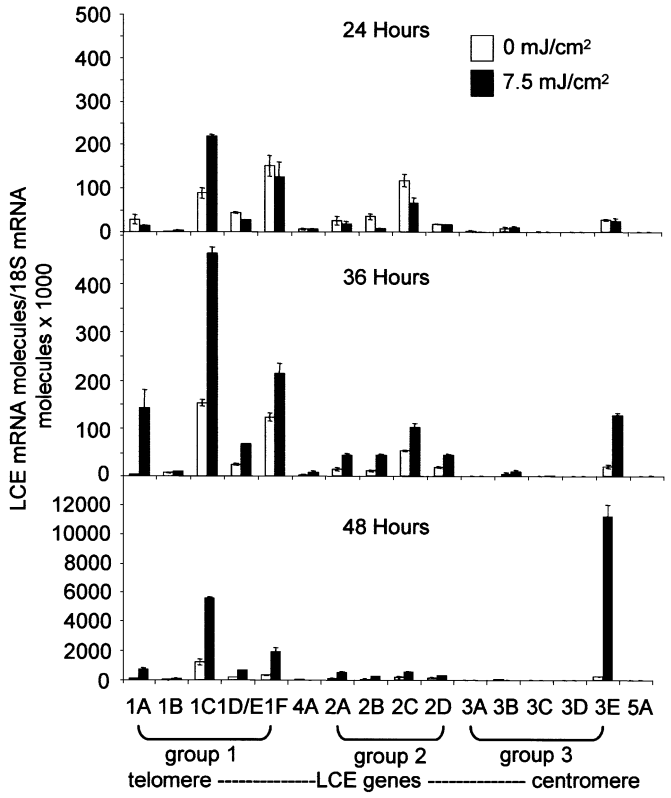


**Figure 7**
**Responsiveness of late cornified envelope (LCE) genes to ultraviolet B (UVB).** (*Top*) 24 h after UV irradiation LCE expression is unchanged, except for LCE1C, which is upregulated. Middle, after 36 h LCE group 1 and 2 genes, and LCE3E show substantial upregulation. LCE3E is the only group 3 gene affected. (*Bottom*) 48 h after UVB irradiation group 1 and 2 genes and LCE3E are strongly upregulated. Note, to accommodate the massive upregulation of LCE3E at 48 h, the scale had to be changed. *Error bars* ± SEM.

UVB irradiation causes upregulation of group 1 and 2 genes, the genes expressed in skin (Fig 4). Upregulation of the dominant skin gene, LCE1C, could be detected at 24 h (Fig 7). By 36 h, all the expressing group 1 and 2 genes (skin genes) were upregulated. Group 3 and 4/5 genes remain expressed at low levels with the sole exception of LCE3E, which alone showed significant upregulation by 36 h and massive and specific upregulation at 48 h after UV irradiation (Fig 7). By 48 h, LCE3E is upregulated over 350-fold, compared with mock-irradiated controls.

## Discussion

This work has been used to link the new LCE gene nomenclature (previously XP5, SPRRL, LEP) to chromosomal structure, gene and protein groupings, gene structures, and expression. LCE genes, with a few key exceptions,

show the canonical two-exon structure associated with many EDC genes, underlying evolutionary relationship. As with previous reports we show chromosomal grouping linked to encoded protein conservation (Marshall *et al*, 2001). Additional to previous reports we show that neighboring NICE-1, a non-LCE member, has regulatory links with LCE3C and that one LCE pseudogene is structurally associated with XP33, another non-LCE gene. The functions of NICE-1 and XP33 are still unknown, as are the functions of C1orf44 and 45, two recently annotated genes in proximity to the LCE cluster (Fig 1, Table I).

**LCE expression and gene-specific *versus* coordinate regulation** We used quantitative analysis to show that the LCE cluster contains two groups of conserved genes (groups 1 and 2) that express predominantly in skin, a third conserved gene group with variable, low-level expression in all stratified, cornifying epithelia, whereas additional LCE 4A and LCE5A genes do not express appreciably under conditions surveyed in this study. We demonstrate group-specific expression in response to changed environmental conditions (calcium and UVB irradiation). This observation is consistent with the idea that proteins characteristic of each LCE group have group-specific functions and/or that gene groups 1 and 2 are under global or coordinate regulation.

We also demonstrate that a single gene from group 3 shows dramatic gene-specific upregulation in response to UVB. Hence, within the LCE group 3, gene-specific regulation is important. As proteins encoded by group 3 are similar this single gene response is compatible with the idea that within-group proteins have similar function and regulatory diversification provides greater potential for responsiveness to environmental challenge (Cabral *et al*, 2001b). According to this theory, the multiplicity of group 3 genes encoding similar proteins may provide an "extended promoter" conferring a greater potential for specific and dramatic change to protein dosage in response to diverse environmental changes. A prediction arising from this theory is that at least some of the genes within any group will encode proteins that are silent or minimally expressed under any particular condition. This prediction is fulfilled for all LCE gene groups. A further untested prediction is that additional LCE genes will behave like LCE3E in response to different types of environment change.

The clustering of EDC genes at 1q21 has long fuelled speculation that either the entire locus, or specific gene clusters, subclusters, or groups are coordinately regulated. Evidence for coordinate regulation of the EDC locus is provided by the demonstration that in interphase keratinocyte nuclei, where EDC genes are transcriptionally active, the EDC adopts a specific subchromosomal location suggestive of chromosomal looping associated with transcriptionally active genes (Williams *et al*, 2002). As with LCE genes, coordinate expression of clustered SPRR and S100 genes has also suggested coordinate regulation (Hardas *et al*, 1996; Patel *et al*, 2003), although there is no direct evidence to date.

On the other hand, evidence for EDC gene-specific regulation is accumulating. As with LCE group 3 genes, it has been shown that specific S100 genes within subclusters are regulated disparately (Elder and Zhao, 2002), demonstrating that local or gene-specific regulation occurs. In addition, a recent SPRR cross-group expression analysis showed that SPRR expression patterns do not reflect structural groupings and led to a conclusion that each gene is under specific regulation (Cabral *et al*, 2001b).

**Response of LCE cluster to UV light** A primary finding of this work is that LCE genes respond to UVB irradiation, thus demonstrating another similarity to SPRR genes. SPRR genes were originally identified as UV responsive genes (Kartasova *et al*, 1988) and it is believed that their UV-dependent upregulation changes the properties of the cornified envelope/stratum corneum.

In this work, LCE induction by UVB had started by 24 h but upregulation of the bulk of the skin genes did not occur until 36 h after irradiation. UV-induced upregulation occurred in response to a dosage of UVB that is physiologically relevant (7.5 J per $m^2$) (Dornelles *et al*, 2004). A similar dosage was used in a recent array analysis of cultured keratinocyte response to UVB (Li *et al*, 2001). This analysis showed waves of transcriptional response at 0.5–2, 4–8, and 16–24 h, with the last wave being associated with changes to cornified envelope precursors. Although SPRR group 2 genes were detected in this study LCE were not, consistent with our demonstration that there is little LCE induction by 24 h. LCE induction represents a longer term skin response to UV.

SPRR4 is also strongly induced by UV (Cabral *et al*, 2001a) and resembles LCE3E in that it appears calcium-independent in culture and is upregulated late (48 h) after UV exposure. UV-induced SPRR4 associates selectively with a subset of cornified envelopes with fragile morphology, providing the best evidence that UV-induced changes to cornified envelope precursors result in physiologically significant cornified envelope changes (Cabral *et al*, 2001a). Acute UV irradiation results in thickening of the stratum corneum and a temporary loss in barrier activity from about 48 h (Haratake *et al*, 1997; Holleran *et al*, 1997) and it is possible that SPRR4 association with UV-induced fragile envelope is part of an attempt to strengthen envelopes to compensate for barrier loss. If so, then SPRR4 should be upregulated in response to other types of barrier disruption. An alternative possibility is that induction of LCE3E and SPRR4 is part of a longer term protective adaption of skin to UV exposure. This interpretation is consistent with the finding that chronic exposure to UV is required for SPRR4 expression *in vivo* (Cabral *et al*, 2001a).

**LCE population heterogeneity** We show in this work that the "skin" pattern of expression is conserved in four individuals assayed at a single skin anatomical site, except that there is heterogeneity in LCE4/5 and LCE2B expression, with one individual lacking LCE4/5 expression. Given that this individual has no major skin defect, this finding shows that ablation of a single LCE gene expression has minimal effect on skin function. This finding also highlights the need for analysis of LCE expression population heterogeneity in conjunction with sophisticated analysis of skin barrier function. It is probable that such population heterogeneity may translate into minor differences in skin barrier function related to subclinical or subtle clinical conditions, e.g., greater

susceptibility to, or differential response to irritants, toxins, or UV irradiation.

## Materials and Methods

**Cell culture** Normal human epidermal keratinocytes (NHEK) from human foreskin were cultured in keratinocyte– serum-free medium (Invitrogen, Paisley, UK) supplemented with epidermal growth factor and bovine pituitary extract. Cells were maintained in 0.09 mM calcium and induced to differentiate in 1.2 mM calcium for 48 h. For UV irradiation experiments cells were grown to confluence to induce differentiation in 0.09 mM calcium, left for 24 h, and then irradiated at 7.5 mJ per $cm^2$ using a UVP CL-1000 UV cross-linker (UVP, Upland, California). Mock-irradiated cells were controls.

**Human skin biopsies** The Medical Ethics Committee of Central Manchester Health Authority approved this research which was performed according to the Declaration of Helsinki Principles. Two healthy females and two healthy males, all aged between 20 and 30 y, agreed to participate and provided written, informed consent. Isopropyl alcohol was used to clean the skin of the left upper inner arm (a non-sun-exposed site) and 1% lignocaine was infiltrated into the skin. One full-thickness 4 mm punch biopsies (Steifel Labs, High Wycombe, UK) of 3 mm depth were taken from the anaesthetized area and hemostasis was maintained for 10 min. Normal skin biopsies from each subject were frozen over liquid nitrogen, and stored at $-80^{\circ}$C.

**Expression analysis** RNA was isolated from NHEK using an RNeasy kit (Qiagen, Crawley, UK) and from human 4 mm arm biopsies using TRIZOL (Invitrogen, UK). Skin (trunk, fetal, penal), vulva, tongue, and esophageal RNA were purchased from Stratagene (La Jolla, California). RNA was converted into cDNA and analyzed by real-time PCR using SYBR Green Core Kit 1 (Eurogentec, Seraing, Belgium) and MJ Research Opticon 1 thermocycler (MJ Research, Waltham, Massachusetts). All reactions were performed in triplicate. Primers (Table S1) were shown to produce a single specific product using product dissociation curves, agarose gel analysis, and DNA sequencing (ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction 3.1 (Applied Biosystems, Foster City, California); Fig S1). Real-time results were calculated relative to human genomic DNA standards (Fig S1). cDNA was shown to be free from genomic contamination by sensitive (35-cycle) PCR using primers that can distinguish cDNA and genomic DNA (TCORF1 primers, Table I; Edwards *et al*, 1997). Real-time results were normalized against keratin 5 (KRT5; tissues), as expression of a basal keratin confined to a single cell layer is expected to be constant in different types of stratified epithelia and will control for varying dermal contamination, and 18S rRNA (keratinocyte culture) because basal keratin expression could diverge between culture experiments. Use of "housekeeping" genes for normalization was avoided following recent criticism (e.g., Bustin, 2002).

**DNA sequencing and analysis** LCE gene 5′ exons were identified using 5′ RACE (Invitrogen), sequenced using ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction 3.1 (Applied Biosystems), analyzed using Biology Workbench software (San Diego Supercomputer Centre, La Jolla, California), and the sequence mapped back to genomic position. The exception to this was LCE3B, whose structure was mapped by sequencing of PCR products and homology to additional LCE group 3 genes.

We would like to thank Dr Ruth Lovering from the HUGO Gene Nomenclature Committee for extensive help with nomenclature reform, Drs C. Backendorf, J. T. Elder, A. Kalinin, and J. A. Segre for helpful comment on nomenclature, and Dr C. Booth for help and advice on analysis. This research was supported by BBSRC BBS/X/00018 to C. Byrne, BBSRC PhD Studentship 01/A2/G/07934 to B. Jackson, and Dr C. Tilli is a Cancer Research UK postdoctoral fellow.

## Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23699/JID23699.htm

**Figure S1** Demonstration of authenticity of real-time PCR strategy.

**Table S1** The DNA sequence of the primers used in real-time PCR analysis with the sizes of the amplified products in base pairs.

DOI: 10.1111/j.0022-202X.2005.23699.x

Manuscript received October 28, 2004; revised December 23, 2004; accepted for publication January 3, 2005

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