Regulation of Epidermal Differentiation by a *Distal-less* Homeodomain Gene

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Abstract. The *Distal-less*–related homeodomain gene *Dlx3* is expressed in terminally differentiated murine epidermal cells. Ectopic expression of this gene in the basal cell layer of transgenic skin results in a severely abnormal epidermal phenotype and leads to perinatal lethality. The basal cells of affected mice ceased to proliferate, and expressed the profilaggrin and loricrin genes which are normally transcribed only in the latest stages of epidermal differentiation. All suprabasal cell types were diminished and the stratum corneum was reduced to a single layer. These data indicate that *Dlx3* misexpression results in transformation of basal cells into more differentiated keratinocytes, suggesting that this homeoprotein is an important regulator of epidermal differentiation.

EPIDERMAL differentiation is a multistage process during which basal keratinocytes cease to proliferate and progressively differentiate as they migrate through the spinous, granular, and cornified layers of the epidermis (Watt, 1989; Fuchs and Byrne, 1994). During this process the cells express specific structural and enzymatic markers that are characteristic and obligatory features of each successive differentiation stage. These markers include the keratin proteins of the intermediate filaments (Steinert and Freedberg, 1991), the intermediate filament–associated protein filaggrin (Dale et al., 1993), and the cell envelope proteins involucrin (Rice and Green, 1979) and loricrin (Hohl and Roop, 1993). Ultimately, activation of keratinocyte-specific transglutaminases leads to a covalent cross-linking of the cornified cell envelope proteins and the formation of the protective stratum corneum (Polakowska and Goldsmith, 1991). The cornified cells, or squames, are the primary barrier protecting the vertebrate body from the environment, and are continually shed, to be replaced by new squames from lower strata. Thus, the epidermis arises from a population of cells that undergo a regular program of differentiation throughout the life of the organism.

The regulation of epidermal specific genes appears to be achieved primarily at the level of RNA synthesis. The transcription of the basal keratins K5 and K14 is markedly reduced in the spinous layer, coinciding with induction of the suprabasal keratins K1 and K10. Subsequently, there is decreased expression of keratins K1 and K10 coupled to the transcriptional activation of the late differentiation markers profilaggrin and loricrin (Fuchs and Byrne, 1994). Several transcription factors have been implicated in the control of specific structural genes expressed in keratinocytes. For some of these, such as AP1, AP2, and KRF-1, binding sites in the regulatory regions of target genes have been identified (Blumenberg, 1993). In addition, other transcription factors have been shown to be present in the epidermis, including the POU family genes Skn1a/i, EPOC-1 and Oct6, and the putative zinc finger factor basonuclin (Andersen et al., 1993; Yukawa et al., 1993; Faus et al., 1994; Tseng and Green, 1992). Some of these have been found to affect gene expression in cultured epithelial cells (Andersen et al., 1993; Faus et al., 1994).

Homeodomain genes, particularly those located in the homeotic complex (HOM-C) of *Drosophila melanogaster*, and their vertebrate orthologues, the Hox genes (McGinnis and Krumlauf, 1992), have been shown to be important in regulating embryonic pattern formation. These genes may play a role in skin development, particularly in its spatially differentiated aspects, such as dorsal/ventral differences or hair patterns. In fact, some of the vertebrate Hox genes are transcribed at significant levels in developing skin (Detmer et al., 1993; Mathews et al., 1993), although the cell type specificity has not been determined for most of these. Homeodomain genes that are not part of the HOM/Hox complexes might also be expected to play a role in skin patterning and cytodifferentiation of epidermis. An example of such a homeodomain gene that is expressed in epidermis is *Distal-less 3 (Dlx3)*.[1]

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1. *Abbreviations used in this paper*: Dlx3, Distal-less 3; PCNA, proliferating cell-specific nuclear antigen.

Distal-less was originally identified in *Drosophila*, where it is important in patterning the legs and sensory appendages (Cohen et al., 1989). The murine *Distal-less* family comprises six individual members, *Dlx1-6* (Robinson et al., 1991; Simeone et al., 1994; Boncinelli, 1994) and interestingly, *Dlx3* differs from the other members of this family because it is not detected in the central nervous system (Boncinelli, 1994). In mouse skin, *Dlx3* is expressed in the suprabasal cells of the interfollicular epidermis and the matrix cells of the hair follicles (Robinson and Mahon, 1994; Morasso et al., 1994). To gain insight into the possible role of the *Dlx3* gene in skin development, we have ectopically expressed the *Xenopus Dlx3* orthologue *(XDlx3)* in mouse basal keratinocytes under the control of a human keratin K5 promoter (Ohtsuki et al., 1992; Byrne and Fuchs, 1993). Ectopic expression of *Dlx3* resulted in profound morphological and biochemical changes in the epidermis. The superficial epidermal phenotype of affected animals was shiny, sticky, and wet. At the histological level, basal cell proliferation was inhibited, and suprabasal cell layers were dramatically reduced. Late differentiation markers loricrin and profilaggrin were severely altered: expression was both reduced in granular cells and ectopically activated in basal cells. Mobility shift and footprinting experiments revealed the presence of a binding site for *Dlx3* in the proximal promoter region of the human profilaggrin gene. These results suggest an important role for *Dlx3* in the process of skin differentiation, which could be exerted in part by a direct interaction between *Dlx3* and regulatory elements of structural genes such as profilaggrin.

Materials and Methods

Transgenic Constructs and Mice

The K5/*XDlx3* construct was generated by subcloning a 905-bp K5 promoter sequence (Ohtsuki et al., 1992; Byrne and Fuchs, 1993) upstream of the coding sequence of the *XDlx3* gene (Dirksen et al., 1994). Downstream of the termination codon, the construct had a segment of the human β globin gene which included the second intron with splice donor and acceptor sites and the polyadenylation sequence. Transgenic mice were generated according to Hogan et al. (1986), and were isolated by cesarean removal at E18.5. Transgenic fetuses and littermates were fixed in 4% paraformaldehyde and skin samples were dissected from the left abdominal area. These samples were embedded in paraffin and sectioned (10 μM).

Immunocytochemistry

Reactions were done with sections of abdominal skin with polyclonal rabbit anti–mouse antibodies against keratins 5, 14, 1, and 10, loricrin, filaggrin; kindly provided by Stuart Yuspa (National Cancer Institute, Bethesda, MD). The secondary antibody was an alkaline phosphatase-conjugated anti–rabbit IgG from Boehringer Mannheim (Indianapolis, IN), and was developed with the BM-purple alkaline phosphatase substrate also from Boehringer Mannheim. Staining of sections with antibody for the proliferating cell nuclear antigen were carried out according to Hansen and Tennant (1994).

In Situ Hybridization

Sense and antisense RNAs were generated for *XDlx3* and mouse loricrin and filaggrin. The construct for RNA synthesis of *XDlx3* included the complete coding sequence for the gene (Dirksen et al., 1994). The construct for loricrin was made by subcloning a 0.62-kb PCR fragment spanning from amino acid 349 to the polyadenylation signal (Mehrel et al., 1990) into pCRII (Invitrogen, San Diego, CA). The filaggrin construct, a 0.3-kb fragment from the coding region, was provided by Dr. S. Yuspa. Digoxigenin-labeled cRNA probes were synthesized in vitro from the corresponding templates for *XDlx3*, profilaggrin and loricrin, and used for hybridization in situ to sections of nontransgenic littermate and transgenic skin according to Schaeren-Wiemers and Gerfin-Moser (1993).

For *Dlx3*, a 1.1-kb cDNA insert (Robinson and Mahon, 1994) was transcribed to generate a [^{35}S]UTP-labeled probe to be used on skin from 2-d-old mice, the slides were coated with emulsion and developed after 5 d exposure (Molecular Histology, Inc., Gaithersburg, MD).

Cornified Envelope Preparation

Cell envelopes were prepared according to Hohl et al. (1991). Briefly, the epidermis was obtained by heat separation from skin samples of normal and affected littermates. The epidermis was stirred gently in extraction buffer containing 100 mM Tris, pH 8.5, 2% SDS, 20 mM DTT and 5 mM EDTA, and boiled for 10 min at 96°C. The cornified envelopes were collected by precipitation at 14,000 rpm for 10 min and reextracted once the same way. After resuspension in the extraction buffer the cornified envelopes were visualized under a phase contrast microscope.

Electron Microscopy

Routine transmission electron microscopy analysis was performed by Advanced Biotechnologies Inc. (Columbia, MD). Briefly, skin samples were fixed in glutaraldehyde, postfixed in osmium tetroxide, stained with uranyl acetate, dehydrated, and embedded in Spurr's plastic resin. The blocks were ultrathin sectioned at a thickness of 60–80 nm, were poststained with lead citrate, and examined under the transmission electron microscope.

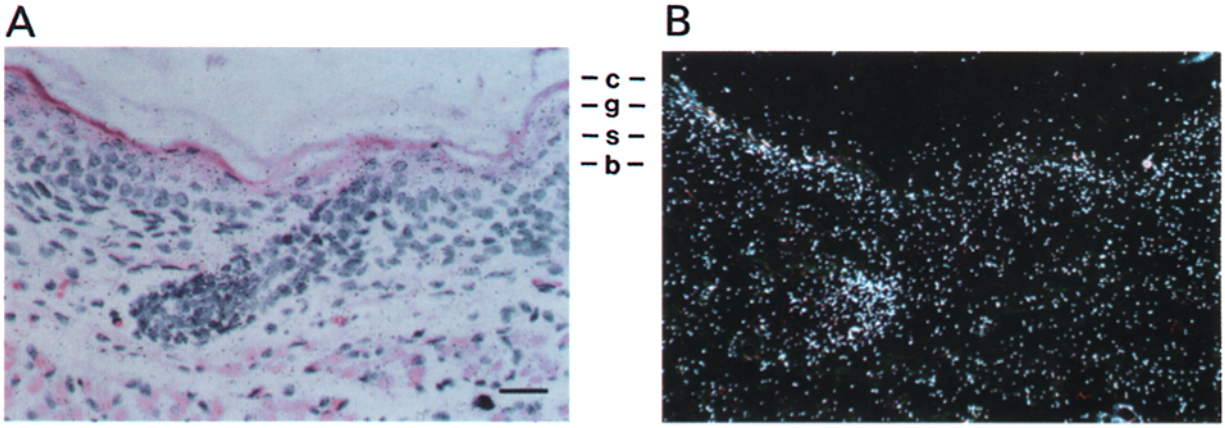

Figure 1. In situ hybridization of *Dlx3* probe to neonatal (2-d) mouse epidermis. Transcripts were detected in the granular cell layer and in the hair follicle matrix cells. (*A*) Bright field with eosin-hematoxylin staining. (*B*) Dark field view of the same section. *c*, cornified; *g*, granular; *s*, spinous; *b*, basal layers. Bar, 20 μm.

Mobility Shift Assays

A recombinant XDlx3 protein was generated by expressing the complete coding sequence for XDlx3 (834 bp), cloned in the EcoRI site of the expression vector pET-28a (Novagen, Madison, WI). Recombinant XDlx3 was purified according to the manufacturer's instructions. Oligonucleotide F1a sequence: CCCTAGGCTTCATTATCTCTTCGAATCCC (profilaggrin promoter sequence underlined); mutated oligonucleotide F1a: CCCTAGGCTTCACTCACAGTTCGAATCCC; TFIID consensus oligonucleotide: GCAGGCATATAAGGTGAGGTAGGA. POU domain consensus oligonucleotide: CTAGAGGATCCATGCAAATGGATCCCCGGGTACCGAGCTC. Binding assays were performed with 1–2 μg of XDlx3 recombinant protein and 4×10^4 cpm (~1 ng) of gel-purified 5′ end-labeled double-stranded DNA fragments. The reactions were carried out in 20 μl containing 10 mM Tris (pH 7.5), 65 mM NaCl, 5 mM DTT, 5 mM $MgCl_2$, 0.05% NP-40, 10% glycerol, 1 mg/ml BSA, and 25 μg/ml poly (dI-dC) as a carrier for 30 min at 4°C. In competition experiments a 100-fold molar excess of the cold competitor was preincubated with the extracts for 30 min at 4°C before adding the labeled DNA fragment. The DNA-protein complexes were resolved on 6% polyacrylamide gels in 0.5× TBE buffer. Gels were dried and exposed overnight at room temperature.

DNase 1 Footprint

Footprints were done on the proximal promoter region of filaggrin with DNaseI/SureTrack Footprinting kit from Pharmacia (Uppsala, Sweden). The DNA probes were amplified by PCR using a 5′ end-labeled oligonucleotide and wild-type or mutated profilaggrin promoter constructs as templates. DNA was gel purified and 1.5×10^4 cpm were used per reaction and treated according to the manufacturer's instructions. The same oligonucleotides were used to amplify a profilaggrin sequence with mutations in the *Dlx3* binding site.

Results

Localization of Dlx3 Expression to Granular Cells

It has been shown previously that *Dlx3* is actively expressed in epidermis (Robinson and Mahon, 1994). Furthermore, this expression is clearly confined to the suprabasal compartment of this tissue, as shown by Northern blot analysis of fractionated epidermis (Morasso et al., 1994). To further sublocalize the expression of *Dlx3*, in situ hybridization of ^{35}S-labeled antisense probe to a section of abdominal 2-d mouse epidermis was carried out (Fig. 1). The highest expression of *Dlx3* was detected in the upper spinous and granular layers, although limited expression in lower spinous cell layers cannot be ruled out. No specific hybridization was detected in parallel sections using sense probe (data not shown).

Targeted Misexpression of Dlx3 to Basal Cells

The restriction of *Dlx3* expression to the suprabasal layers, predominantly to the granular cells, raised the possibility that this homeodomain protein might be involved in the regulation of the later stages of epidermal differentiation. To test this hypothesis, we targeted *Dlx3* transcription to the basal keratinocytes of mouse skin by expressing the Xenopus orthologue of *Dlx3* under the control of a 905-bp 5′ flanking element from the human keratin K5 gene (Fig. 2 *A*). In transfection experiments, Ohtsuki et al. (1992) have demonstrated that this 905-bp element was preferentially active in epithelial cells. In transgenic studies, Byrne and Fuchs (1993) showed that a 6.5-kb human K5 region encompassing the 905-bp sequence was faithfully expressed in mouse epidermis. As shown in Fig. 2 *C*, the 905-bp K5 fragment was uniformly active only in the basal cell layer,

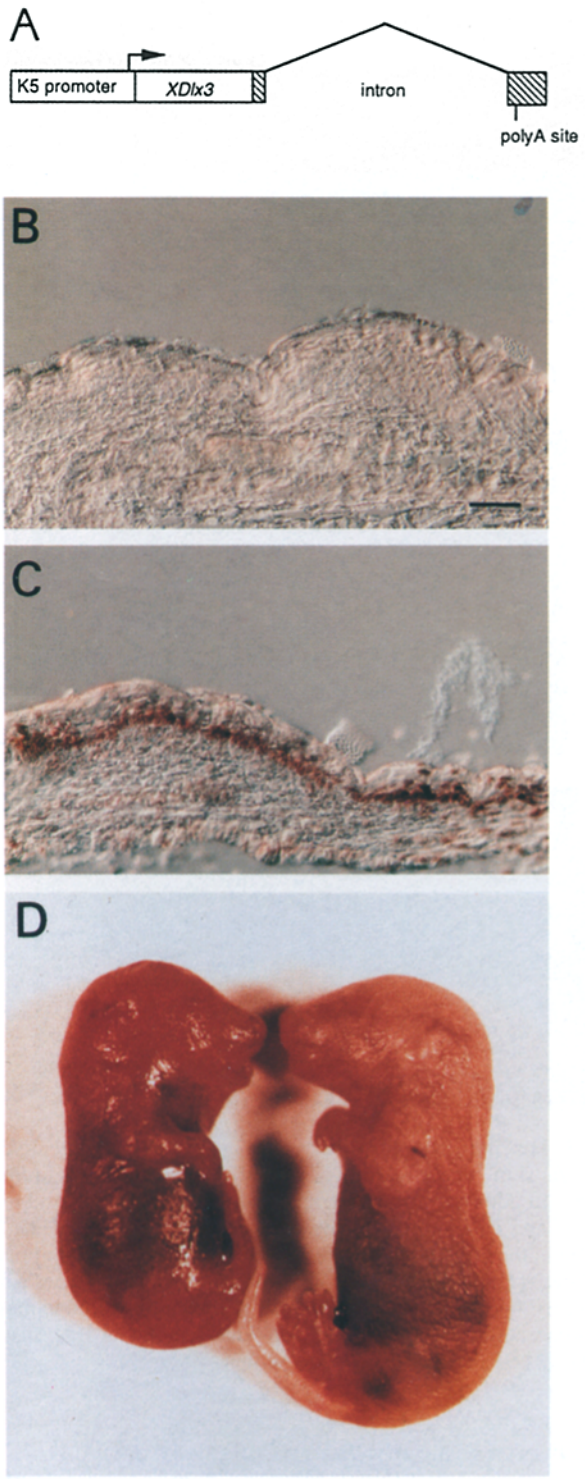

Figure 2. Transgene construct, expression, and gross phenotype. (*A*) K5-*XDlx3* transgenic construct. Hybridization of sense (*B*) and antisense (*C*) *XDlx3* probe to abdominal skin sections (10 μM) of affected transgenic E18.5 embryos. Expression was confined to the basal layer. (*D*) Appearance of severely affected K5/*XDlx3* transgenic embryo (*left*) and normal littermate (*right*). Bar, 40 μm.

indicating that this region contains the necessary elements to restrict the epidermal expression of the *Xenopus Dlx3* gene to the basal cells in transgenic animals. Hybridization was also detected in hair follicles (data not shown). The

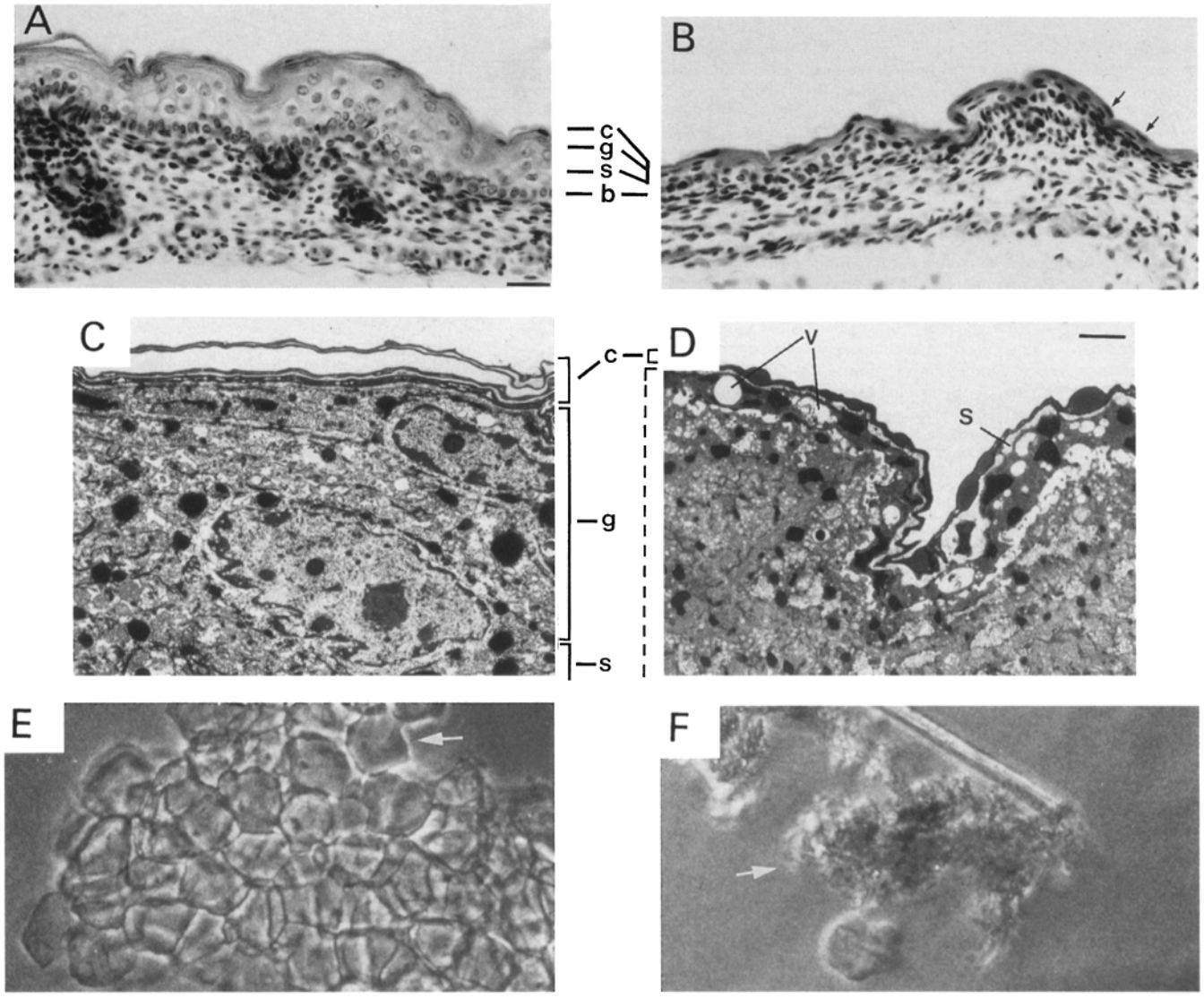

Figure 3. Histology and ultrastructure of normal and transgenic E18.5 epidermis. (*A* and *B*) Hematoxylin staining of normal (*A*) and affected (*B*) skin. The thickness of the differentiated layers in the transgenic skin was substantially diminished when compared to normal skin. In addition, basal layer cells appeared flattened, and many nucleated cells were seen in the upper strata of affected skin (*arrows*). (*C* and *D*) Electron microscopy of normal (*C*) and affected transgenic (*D*) epidermis, showing a dramatic reduction of the stratum corneum in the affected skin. The affected stratum corneum appeared to be partially separated from underlying cells, which were highly vacuolated. (*E* and *F*) Preparations of cornified cell envelopes isolated from control (*E*) and transgenic (*F*) skin. Arrow in *E* indicates a typical cornified envelope. In *F*, the arrow indicates insoluble material isolated from affected skin. *V*, vacuoles; *S*, separation of stratum corneum. *c*, cornified; *g*, granular; *s*, spinous; *b*, basal layers. In *D*, granular and spinous cell morphology is abnormal (*dashed line*). Bars: (*A*) 20 μm; (*D*) 1.5 μM.

Xenopus orthologue of *Dlx3* was originally named *Xdll2* (Dirksen et al., 1994; Papalopulu and Kintner, 1994), but to avoid confusion we will refer to this gene as *XDlx3*. *XDlx3* and murine *Dlx3* have identical homeodomains, except for two conservative substitutions in helix 1. All of the nonhomeodomain homology elements are highly conserved between the two genes. Furthermore, the expression patterns of *XDlx3* and *Dlx3* are very similar in frog and mouse (Robinson and Mahon, 1994; Dirksen et al., 1994), and as we have reported recently, the regulatory elements of the *Xenopus* gene function in transgenic mice to produce an expression pattern essentially identical to that of the endogenous mouse *Dlx3* (Morasso et al., 1995).

Phenotype of Transgenic Mice

Transgenic mice expressing the K5/*XDlx3* construct tended to be smaller, but did not display obvious body or limb deformities, and were of similar developmental stage compared to the unaffected siblings, as judged from anatomical features such as digit morphology (data not shown). Affected mice could be immediately recognized by highly abnormal skin, which was partially transparent, shiny, sticky, and wet suggesting a disturbed barrier function (Fig. 2 *D*). The phenotype was variable in severity, and moderately affected embryos showed a more pronounced phenotype in the ventral epidermis than on the dorsal side. Severely

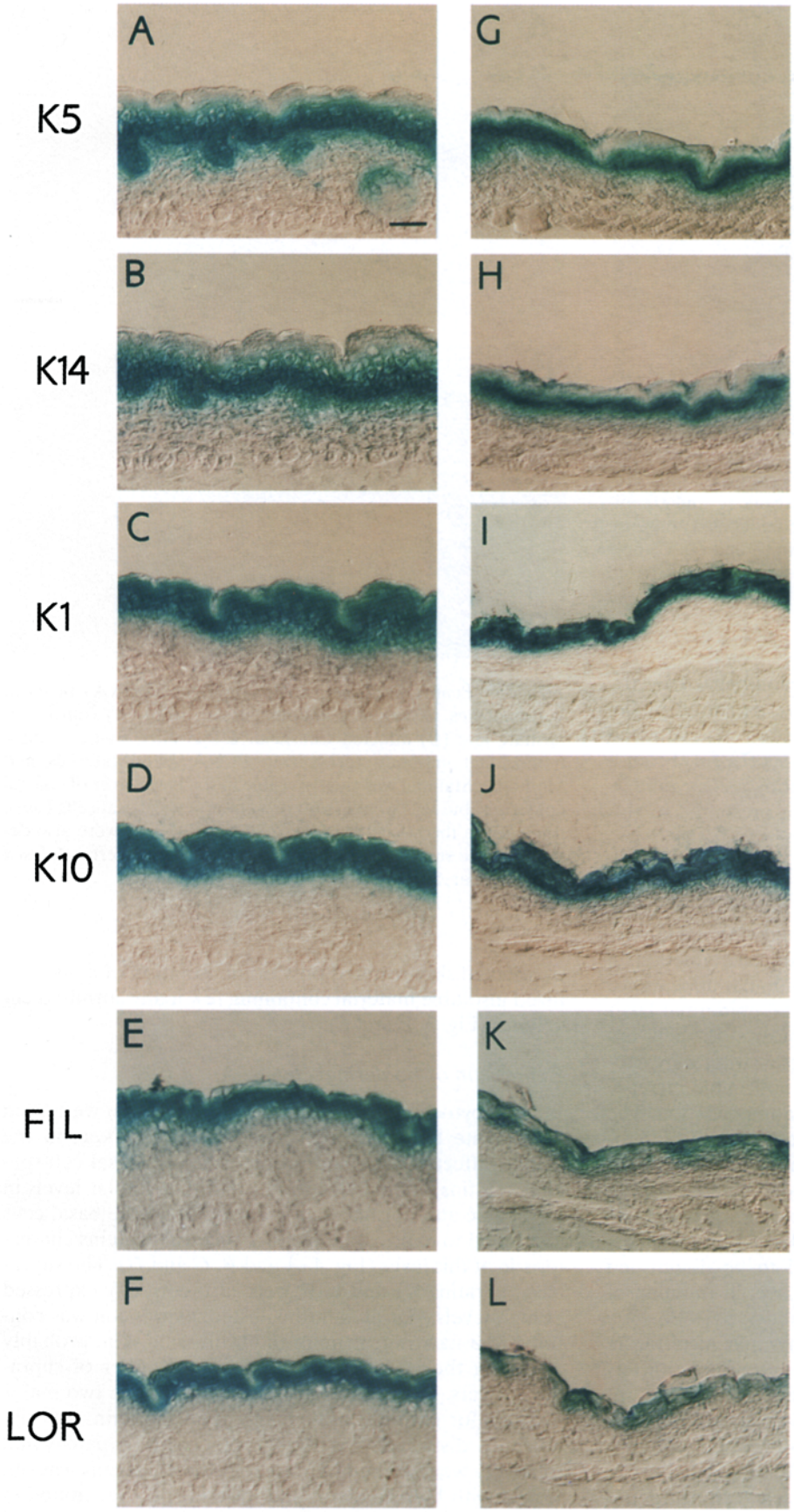

Figure 4. Immunocytochemistry of epidermal markers in E18.5 abdominal skin from normal and transgenic mice. Sections from normal littermate (*A–F*) and transgenic (*G–L*) mice were stained with anti-keratin 5 antibody (K5; *A* and *G*), anti keratin 14 antibody (K14; *B* and *H*), anti-keratin 1 antibody (K1; *C* and *I*), anti-keratin 10 antibody (K10; *D* and *J*), anti-filaggrin antibody (FIL; *E* and *K*) and anti-loricrin antibody (LOR; *F* and *L*). The staining of keratins was relatively unchanged with respect to localization and intensity (*A–D*, *G–J*), whereas staining for filaggrin (*E* and *K*) and loricrin (*F* and *L*) was markedly reduced in the granular layer of the affected compared to the nontransgenic epidermis. Bar, 40 μm.

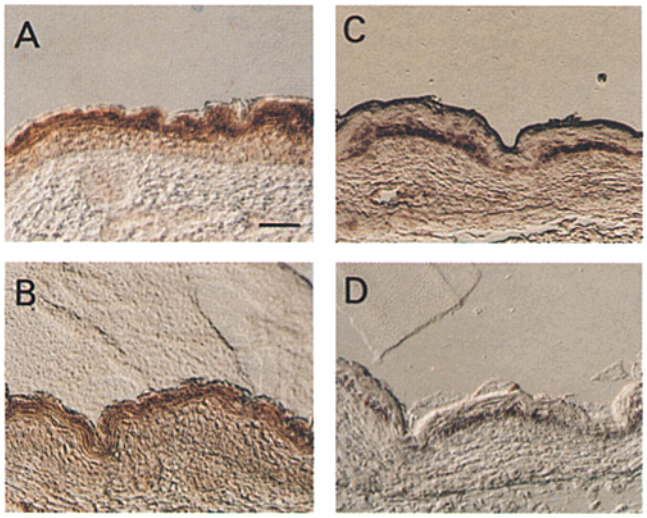

Figure 5. Detection of profilaggrin and loricrin transcripts. Sections of normal (*A* and *B*) and affected transgenic (*C* and *D*) epidermis were hybridized in situ with digoxigenin-labeled antisense probes for profilaggrin (*A* and *C*) and loricrin (*B* and *D*). Ectopic expression of both genes was visible in the basal cells of affected epidermis (*C* and *D*). Bar, 40 μm.

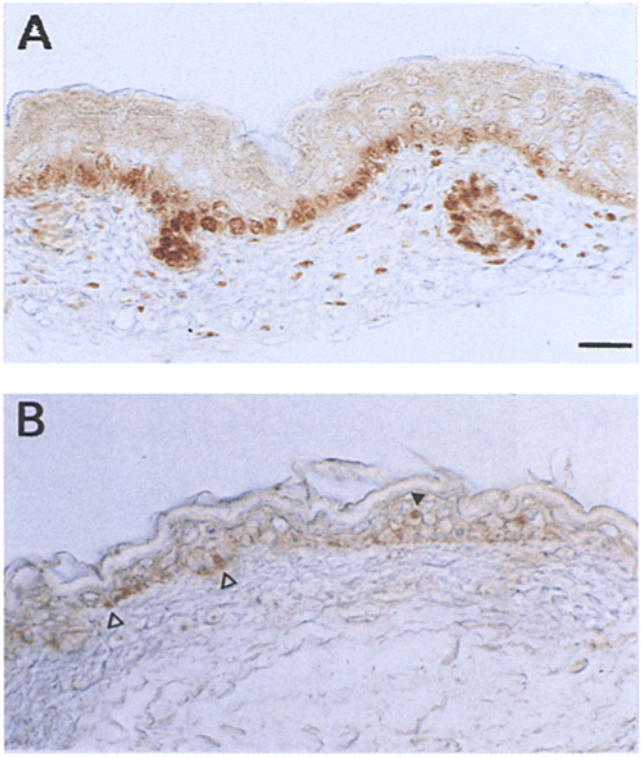

Figure 6. Proliferating cell nuclear antigen (PCNA) in basal keratinocytes. Sections of abdominal skin from (*A*) control littermate and (*B*) affected transgenic E18.5 mice stained with a monoclonal antibody recognizing PCNA (see Materials and Methods). Staining was clearly visible in the basal layer of control epidermis, but was weaker and present in fewer basal cells (*open triangles*) in the affected skin. PCNA-positive cells were also detected in the suprabasal strata of the transgenic epidermis (*closed triangle*). Bar, 20 μm.

affected neonates were immediately cannibalized by the mothers. To circumvent this, pups were delivered by cesarean section at about E18.5. Attempts to rescue affected animals with foster mothers were not successful. From a total of 96 pups examined, eight exhibiting a clear epidermal phenotype were studied. The data presented in this paper were derived from severely affected animals.

Histology

Sections of E18.5 abdominal skin, stained with hematoxylin (Fig. 3), clearly revealed a pronounced reduction in skin thickness. Instead of the typical columnar appearance in normal epidermis (Fig. 3 *A*), the basal epidermal layer of affected skin was disorganized and most of the cells appeared flattened (Fig. 3 *B*). All suprabasal strata were substantially diminished, and there was a noticeable increase of nucleated (parakeratotic) cells in the upper layers. The number of hair follicles was reduced from 5.1 ± 0.4 (75 total) to 1.2 ± 0.3 (26 total) follicles/mm in sections of normal vs affected epidermis. There was no evidence for lymphocyte infiltration or other inflammatory responses in the affected epidermis or underlying dermis. In most sections, the dermal layer also appeared to be diminished compared to controls, resulting in an overall thinning of the skin.

Fig. 3, *C* and *D* show electron micrographs of ultrathin abdominal skin sections from normal and affected littermates, respectively. The most striking abnormality induced by *XDlx3* misexpression was in the stratum corneum, which was reduced from the multilayered structure of normal epidermis to a single layer. This abnormal cornified layer appeared to be partially detached from the underlying cells, which were highly vacuolated. Cornified envelopes can be isolated from skin by extraction with SDS at high temperature (Hohl et al., 1991). Such an extraction of affected skin resulted only in the isolation of a disorganized insoluble material containing few if any cornified envelopes (Fig. 3, *E* and *F*).

Expression of Epidermal Genes

Immunocytochemistry and in situ hybridization were used to evaluate the expression of epidermal markers in the skin of affected mice and normal siblings. Basal cell-specific keratins K5 and K14 were present at similar levels in transgenic and normal skin, indicating that the basal cells continued to express the major structural proteins characteristic of this layer (Fig. 4, *A* and *B*, *G* and *H*). The suprabasal keratins K1 and K10, were also correctly expressed at high levels. For all keratins tested, expression was confined to a narrower zone in the transgenic skin, probably reflecting the general decrease in the number of suprabasal layers. In contrast, the expression of the two major markers for granular cells, filaggrin and loricrin, was significantly disturbed in transgenic epidermis. A patchy and relatively weak staining throughout the epidermis was observed with both filaggrin and loricrin-specific antibodies (Fig. 4, *E*, *F*, *K*, and *L*). This indicated that misexpression of *XDlx3* resulted in both downregulation of filaggrin and loricrin in granular layer cells and ectopic synthesis of these

proteins in lower strata. To determine whether these effects were due to deregulated transcription, in situ hybridization was performed. The hybridization of antisense profilaggrin and loricrin RNA probes is shown in Fig. 5. Comparison of these results revealed that while profilaggrin (Fig. 5 *A*) and loricrin (Fig. 5 *B*) transcripts were uniformly and abundantly expressed in the granular cells of unaffected siblings, only a few isolated cells expressing these genes were detected in the granular layers of the transgenic skin (Fig. 5, *C* and *D*). However, profilaggrin and loricrin transcripts were expressed in the basal cells and in the lowest spinous layer.

In normal epidermis, cell division is primarily confined to the basal layer (Watt, 1989; Fuchs and Byrne, 1994). Although the transgenic basal cells expressed the K5 and K14 markers, the perturbance of the typical basal layer configuration and the flattened morphology suggested that these cells might have acquired a more differentiated character, one aspect of which is the cessation of proliferation. To evaluate this, sections were stained with an antibody recognizing the proliferating cell-specific nuclear antigen (PCNA; Hansen and Tennant, 1994). This antigen is most abundant at S phase, although it is also found in other phases of the cell cycle (Kurki et al., 1986). As shown in Fig. 6, this marker was detected at much higher levels in nontransgenic basal cells compared to the epidermis of affected mice, indicating that *XDlx3* misexpression correlated with the suppression of basal cell proliferation. Interestingly, PCNA staining in the dermis of affected skin was also drastically reduced compared to controls.

Interaction of XDlx3 with the Profilaggrin Promoter Region

In normal epidermis, the profilaggrin and loricrin genes become activated in the granular layer, in the same cells in which *Dlx3* is maximally expressed. In view of this correlation, one possible explanation for the ectopic activation of profilaggrin and loricrin in basal cells forced to produce the XDlx3 protein could be that one or both genes were directly regulated by this homeoprotein. To investigate this possibility, bacterially expressed XDlx3 protein was used in mobility shift and footprinting studies with the proximal promoter of the profilaggrin gene. This region has been shown to be sufficient to confer high levels of keratinocyte-specific transcription to a reporter gene in cultured cells (Jang et al., 1996). A series of overlapping oligonucleotides spanning the 120-bp proximal promoter of the profilaggrin gene (Fig. 7 *A*) was tested for binding to recombinant XDlx3 homeoprotein.

From these probes (indicated in Fig. 7 *A*), the oligonucleotide F1, which contains an AT-rich potential homeodomain-binding motif (Gehring et al., 1994), was found to produce two complexes (data not shown). A smaller oligonucleotide (F1a) containing only this putative binding site, yielded an identical mobility shift pattern (Fig. 7 *B*). Both of the shifted bands (lane *2*, *arrowheads*) were completely eliminated by competition with unlabeled probe (lane *3*). Competition was not observed with oligonucleotides in which the AT-rich core nucleotides had been mutated (lane *4*) or with a consensus binding site for POU domain proteins (lane *5*) or an unrelated AT-rich sequence, the con-

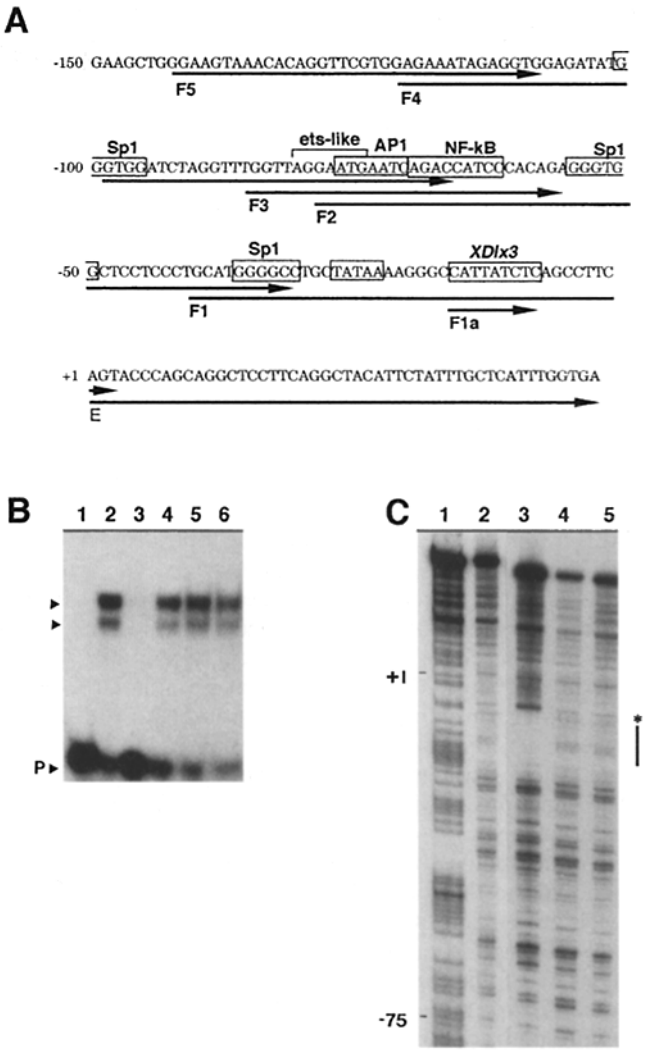

Figure 7. Detection of a XDlx3 binding site in the profilaggrin proximal promoter. (*A*) Sequence of the proximal promoter region of the profilaggrin gene. The specific oligonucleotides used in mobility shift assays (F1-F5, F1a, E) are indicated by the arrowed lines below the sequence. Functional transcription factor binding sites are indicated, including the region protected from DNase I by binding to XDlx3. (*B*) Mobility shift analysis. 1–2 μg of recombinant XDlx3 were combined with end-labeled double-stranded oligonucleotide under the conditions described in Materials and Methods. Lane *1*, oligonucleotide probe F1a alone. Lane *2*, probe F1a with recombinant XDlx3 protein. Lanes *3–6*, competition with a 100-fold molar excess of unlabeled oligonucleotide F1a, mutated oligonucleotide F1a, Oct consensus sequence oligonucleotide, and TFIID consensus oligonucleotide, respectively. P, probe. Shifted complexes are indicated by triangles. (*C*) DNaseI footprinting assay. An end-labeled DNA fragment encompassing the profilaggrin gene region between −150 and +47 was incubated with recombinant XDlx3 and treated with DNaseI. Lane *1*, G+A ladder. Lane *2*, no XDlx3 protein added. Lane *3*, XDlx3 footprint. Lane *4*, competition with a 100-fold molar excess of oligonucleotide F1a. Lane *5*, footprinting reaction carried out with a profilaggrin probe in which the AT-rich XDlx3 binding site was mutated (see Materials and Methods). The protected bases are indicated by the vertical bar, and a hypersensitive site by an asterisk. Position of profilaggrin gene base pairs +1 and −75, corresponding to *A*, are indicated to the left.

sensus TFIID motif (lane *6*). These results were confirmed by DNaseI footprint analysis, and the results are shown in Fig. 7 *C*. A single protected region was detected that coincided with the sequence of the probe used in the bandshift analysis (CATTATCTC; lane *3*). The protection was abolished when the binding assay was done in the presence of a 100-fold excess of unlabeled oligonucleotide F1a (lane *4*), or when mutations identical to those used in Fig. 7 *B* were introduced in the site (lane *5*).

Discussion

In this study we show that the misexpression of the suprabasal cell-specific homeobox gene *XDlx3* in the basal cells of transgenic mice has two important effects. First, the targeted basal keratinocytes are transformed into a more differentiated phenotype. Second, the overall structure of the epidermis, particularly of the more differentiated strata, is severely disrupted. As a result of ectopically expressing *XDlx3* in basal cells, the granular cell phenotype is prematurely initiated, as judged by the cessation of cell division and the activation of the genes encoding the major structural proteins characteristic of this cell type, filaggrin and loricrin. We show that the proximal promoter region of the profilaggrin gene includes a binding site for XDlx3, suggesting a direct interaction between XDlx3 and this gene. These findings strongly support the conclusion that Dlx3 functions in vivo to regulate the differentiation of epidermal cells.

It is instructive to compare the *XDlx3* misexpression phenotype to that of epidermis in which other regulators of cell growth and differentiation have been altered. The basal-cell targeted expression of lymphoid enhancer factor 1 affects hair follicle and tooth formation, but does not otherwise disrupt the epidermal differentiation or proliferation programs (Zhou et al., 1995). Another example is the overexpression of cyclin D1 using a bovine keratin K5 promoter, which results in hyperproliferation, but does not interfere with the differentiation of stratified epithelia (Robles et al., 1996).

Especially interesting is the comparison of the *XDlx3* misexpression phenotype to that in which retinoid signaling has been disrupted by dominant negative retinoic acid receptors driven by K14 or K1 promoters. Targeting such molecules to basal cells resulted in thinning of the epidermis, delay in hair follicle formation, and virtual elimination of filaggrin gene expression (Saitou et al., 1995). Expression of a similar dominant negative retinoic acid receptor in suprabasal cells resulted in abnormal lipid deposition and concomitant disruption of the epidermal barrier function, but did not lead to alterations in the cell type specificity of gene expression (Imakado et al., 1995). Thus, retinoid signaling appears to function in the coordination of epidermal development, whereas Dlx3 seems to more directly control the spectrum of gene expression in suprabasal cells.

How does misexpression of *XDlx3* in basal keratinocytes result in the pathological phenotype of the epidermis? Affected animals had reduced levels of filaggrin and loricrin in the granular cells. These are the two major proteins of the differentiated keratinocyte, and are thought to play important roles in the alignment of keratin filaments and formation of the moisture barrier provided by the cornified cell envelope (Dale et al., 1978; Mehrel et al., 1990). Judging from the phenotype observed when these genes are down-regulated in skin disorders, it is unlikely that the reduced levels of these proteins alone can account for the morphology of affected skin. For example, congenital genodermatosis icthyosis vulgaris is accompanied by a drastic reduction in the number of keratohyalin granules, and in many patients no profilaggrin mRNA or protein can be detected (Nirunsuksiri et al., 1995). Histologically the disease is characterized by hyperkeratosis rather than reduction of the cornified layers and the symptoms are very mild compared to the disruption of epidermis resulting from *XDlx3* misexpression. Therefore the severe skin phenotype in the *XDlx3* transgenic mice probably has a more complex explanation.

One possibility is that affected basal cells are physically disrupted by accumulation of ectopic profilaggrin and/or loricrin, leading to secondary defects. Support for this interpretation comes from observations indicating that inappropriate expression of epidermal structural proteins interferes with the normal function of the keratinocyte. For example, Carroll et al. (1995) have reported that prolonging integrin gene expression beyond the basal-suprabasal transition, by forcing ectopic expression via an involucrin gene promoter, results in an abnormal epidermal phenotype resembling psoriasis. Also, inappropriate expression of suprabasal keratins K1 and K10 interferes with the proliferation of keratinocyte-derived tumor cell lines, suggesting that the incorporation of these proteins into the intermediate filament cytoskeleton is incompatible with mitosis, a key feature of cells in the basal compartment (Kartasova et al., 1992). Thus, it is possible that premature expression of the profilaggrin gene might itself be the basis for much of the observed epidermal dysplasia. Forced expression of filaggrin in cultured cells can lead to collapse of the intermediate filament network, disruption of the nuclear membrane, vacuolization, and eventually cell death (Dale et al., 1997; L.-G. Kim, L.-S. Park, and P.M. Steinert, personal communication). The primary effect of XDlx3 could be limited to activation of profilaggrin expression in basal cells, while the other features of affected skin are secondary consequences of the presence of this protein.

A second alternative to account for the skin abnormalities is that misexpression of *XDlx3* could result in a general and premature activation of keratinocyte differentiation, including suppression of cell division which reduces the supply of cells entering the suprabasal compartment. Premature initiation of the granular cell differentiation program at the basal cell level could make it impossible to properly organize the structural elements required for cornified cell formation. Differentiation could also be fundamentally incompatible with basal cell proliferation, reducing the supply of cells capable of migration into the suprabasal strata. One argument in favor of this interpretation comes from the fact (Fig. 6) that little PCNA staining was observed in the basal layer of affected skin.

The differentiation process by which the epidermal keratinocyte is transformed from a columnar mitotically active basal cell into the flattened anucleated cornified cell has only begun to be understood. The data presented in this paper show that *Dlx3* plays an important role in mediating this program.

We thank Drs. J. Compton, A. Dlugosz, J. Franklin, C. Nocente, P. Steinert, and S. Yuspa for helpful discussions and invaluable assistance and advice in this work; Dr. S. Yuspa for supplying the epidermal antibodies and filaggrin construct; and Dr. M. Blumenberg for providing us with the K5 promoter region. We are grateful to Drs. B. Dale, R. Presland, R. Lewis, P. Fleckman, L.-G. Kim, L.-S. Park, and P. Steinert for sharing their unpublished results.

Received for publication 15 September 1996 and in revised form 29 October 1996.

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