Cloning and Characterization of the Human Protein Kinase C-\(\eta\) Promoter*

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Protein kinase C-\(\eta\) (PKC-\(\eta\)) is predominantly expressed in epithelial tissue, including lung, intestine, and skin. In skin, PKC-\(\eta\) expression is limited to keratinocytes in the upper layers of the epidermis. To investigate regulation of cell type-specific expression of PKC-\(\eta\), we cloned the 5'-segment of the PKC-\(\eta\) gene from a P1 genomic library. A 9.4-kilobase pair fragment encompassing the 5'-flanking region, first exon, and first intron, was localized on human chromosome 14 (14q22–23). Two major transcription initiation sites identified by reverse transcriptase polymerase chain reaction, primer extension, and S1 nuclease mapping, were located approximately 650 base pairs upstream from the translation start site. The human PKC-\(\eta\) proximal promoter region lacks canonical TATA and CAAT boxes and GC-rich regions. A 1.6-kilobase pair 5'-flanking region displayed maximal promoter activity. This promoter was active in human keratinocytes but not human skin fibroblasts, in accord with endogenous PKC-\(\eta\) gene expression. Stepwise 5' deletion analysis revealed the presence of adjacent regulatory regions containing silencer and enhancer elements located 1821–1702 base pairs and 1259–1189 base pairs upstream of the transcription initiation site. Deletion of the proximal PKC-\(\eta\) promoter rendered the enhancer element inactive. Both the silencer and enhancer elements regulated heterologous promoters in keratinocytes but not fibroblasts. Electrophoretic mobility shift analysis demonstrated specific protein binding to Ets/heat shock factor and Ets/activator protein-1 consensus sequences in the enhancer and silencer regions, respectively. Mutations of the Ets/heat shock factor binding sites caused loss of functional enhancer activity. These data elucidate transcriptional regulation and tissue-specific expression of the PKC-\(\eta\) gene.

The protein kinase C (PKC)\(^3\) group is a large family of phospholipid-dependent serine/threonine protein kinases that are involved in a wide variety of cellular processes, including membrane receptor signal transduction, control of gene expression, and cell proliferation and differentiation (1–3). Recent molecular cloning studies have revealed that the PKC family consists of at least 11 distinct isoforms, which have been categorized into three subgroups based on their structure and cofactor requirements. Conventional PKC members (\(\alpha\), \(\beta\), and \(\gamma\)) are activated by diacylglycerol, acidic phospholipid, and Ca\(^{2+}\). Novel PKC members (\(\delta\), \(\epsilon\), \(\eta\), \(\theta\), and \(\mu\)) are activated by diacylglycerol and acidic phospholipid but are not dependent on Ca\(^{2+}\). Atypical PKCs (\(\zeta\) and \(\lambda\)) are not stimulated by either diacylglycerol or Ca\(^{2+}\) (4). PKC isoforms show distinct tissue, cellular, and subcellular distributions, and individual cells often express several isoforms (5–7). The presence of multiple isoforms, their differential expression in mammalian tissues, and their different cofactor requirements suggest that different PKC isoforms may be regulated independently and may have different biological functions in signal transduction processes.

Keratinocytes, the predominant cell type in human skin, express at least five PKC isoforms: \(\alpha\), \(\delta\), \(\eta\), \(\zeta\), and \(\lambda\) (5). In situ hybridization and immunohistochemical staining indicate that PKC-\(\eta\) expression in human skin is restricted to keratinocytes undergoing terminal differentiation in the outermost layers of the epidermis (8). During this terminal differentiation, keratinocytes that express PKC-\(\eta\) synthesize insoluble cross-linked envelopes and exude complex lipids that together form the structural basis of the skin barrier (9). PKC-\(\eta\) expression is highly tissue-specific; it is expressed predominantly in epithelial tissues such as skin, lung, and intestine (10–12). This limited distribution of PKC-\(\eta\) contrasts with the ubiquitous expression of other PKC isoforms in human tissue (5).

The restricted expression of PKC-\(\eta\) to differentiating skin keratinocytes and additional evidence suggest that PKC-\(\eta\) may function to regulate keratinocyte terminal differentiation (9, 13). If so, regulation of PKC-\(\eta\) expression may be critically important for the formation and maintenance of the human skin barrier, which is necessary for life. In view of these considerations, we investigated regulation of PKC-\(\eta\) gene expression. In this study we describe the cloning, chromosomal localization, and functional characterization of the human PKC-\(\eta\) promoter.

EXPERIMENTAL PROCEDURES

Isolation of a PKC-\(\eta\) Genomic Clone—A P1 genomic library (Genome Systems Inc., St. Louis, MO) was initially screened using a 125-bp cDNA probe that corresponded to the 5'-end of the published PKC-\(\eta\) partial cDNA (11). The cDNA probe was random-labeled with [\(\alpha\]\(^{32}\)P]dCTP (NEN Life Science Products, Boston, MA). Prehybridization was performed at 50 °C for 1 h; hybridization was then carried out at 50 °C overnight, as described (14). This process yielded a positive 80-kb genomic clone designated 7239. This large P1 genomic clone was digested with BamII and HindIII, and the resulting fragments were subcloned into the BamHI and HindIII sites of cloning vector pZErO-1 (Invitrogen, San Diego, CA). The pZErO-1 subclones were rescreened.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) AF045569.

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The abbreviations used are: PKC, protein kinase C; kb, kilobase pairs; bp, base pairs; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; RT, reverse transcriptase; Spi, stimulatory protein 1; HSF, heat shock factor; AP-1, activator protein-1; PIPES, 1,4-piperazineethanesulfonic acid; EMSA, electrophoretic mobility shift assay; DAPI, 4,6-diamidino-2-phenylindole.

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with the 125-bp probe, yielding 15 positive genomic clones of which two were unique, pTHQ5 and pTHQ15. To obtain additional 5′-upstream sequence, an EcoRI, XbaI sublibrary was generated from the P1 genomic clone, as described above, and screened with a 110-bp probe corresponding to the 5′-end of pTHQ15. Twenty-one additional positive clones were identified and visualized with the 32P-labeled probe, pTHQ35. The alignment of these four unique clones was determined by restriction mapping (BamHI, HindIII, EcoRI, and XbaI), PCR analysis, nucleotide sequencing, and Lasergene computer program (DNASTar Inc., Madison, WI).

DNA Sequence Analysis—All oligonucleotides were synthesized by the Biomedical Research DNA Core Facility (University of Michigan, Ann Arbor, MI). Nucleic acid sequences of both strands were determined by automated sequencing using an applied Biosystems DNA Sequencer (model 377) or manually by the dideoxy chain termination method (15). Nucleotide sequences were confirmed by manual sequencing using a SequiTherm cycle sequencing kit (Epicentre Technologies, Madison, WI). Sequences spanning the transcription initiation site and exon-intron boundaries were subcloned into TA cloning vectors (pCR2.1, Invitrogen, San Diego, CA) and subjected to manual sequencing in both directions, as described above. To identify putative cis-regulatory elements, 3.6 kb of 5′-flanking DNA of the human PKC-γ gene was analyzed with transcription element search software (Computational Biology and Informatics Lab, School of Medicine, University of Pennsylvania, Philadelphia, PA).

Cell Culture—Human keratinocyte HaCaT cells and primary human fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Primary human skin fibroblasts were as described previously (5), and used at passage three or four. Keratinocyte and fibroblast cultures were established from normal human skin, as described previously (5), and used at passage three or four.

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RNA Isolation and Nucleotide Protein Extracts—Total RNA was purified from human skin by the guanidinium/cesium trifluoroacetic acid hypotonic buffer (1 M HEPES, pH 7.9, 1 M KCl, 1 M MgCl2, 1 M dithiothreitol, 5 M NaCl, 5 M PIPES, pH 6.5, at 55 °C overnight. S1 nuclease (100 units, Roche Molecular Biochemicals) was then added to the digestion buffer (280 mM sodium chloride, 30 mM sodium acetate, 4.5 mM zinc acetate, and 20 µg/ml of salmon sperm DNA) for 30 min at 37 °C. Remaining DNA-RNA hybridized fragments were analyzed as described above for primer extension analysis.

Electrophoretic Mobility Shift Assays—EMSA probe were used for a gelshift assay (Stratagene, La Jolla, CA) with minor modifications. 200 µg of nuclear extract (40% of a 10–50-fold molar excess of cold competitor double-stranded oligonucleotide) was preincubated with nuclear extracts for 30 min at room temperature. Preincubated complexes were added to 10 µl of 5X gel loading buffer (90% formamide, 5 mM EDTA, 0.05% bromphenol blue, 0.05% xyleneol, boiled for 5 min, chilled on ice, and loaded on a 6% acrylamide/8 M urea sequencing gel. The gel was transferred to 3 mm Whatman paper, dried, and scanned with a PhosphorImager (Molecular Dynamics). Nuclease digestion of genomic DNA was performed using 25 units of micrococcal nuclease (New England Biolabs, Beverly, MA) for 15 min at 37 °C. The digestion mixture was then treated with 5% SDS and extracted with phenol/chloroform. The digest was ethanol precipitated and resuspended in TE to a final concentration of 2 µg/µl. This DNA was used as a control for S1 nuclease digestion. A gel containing 25 µg of sample DNA in each lane was run on a 1% agarose gel and stained with ethidium bromide. The gel was scanned with a PhosphorImager (Molecular Dynamics). Nuclease digestion of genomic DNA was performed using 25 units of micrococcal nuclease (New England Biolabs, Beverly, MA) for 15 min at 37 °C. The digestion mixture was then treated with 5% SDS and extracted with phenol/chloroform. The digest was ethanol precipitated and resuspended in TE to a final concentration of 2 µg/µl. This DNA was used as a control for S1 nuclease digestion. A gel containing 25 µg of sample DNA in each lane was run on a 1% agarose gel and stained with ethidium bromide. The gel was scanned with a PhosphorImager (Molecular Dynamics). Nuclease digestion of genomic DNA was performed using 25 units of micrococcal nuclease (New England Biolabs, Beverly, MA) for 15 min at 37 °C. The digestion mixture was then treated with 5% SDS and extracted with phenol/chloroform. The digest was ethanol precipitated and resuspended in TE to a final concentration of 2 µg/µl. This DNA was used as a control for S1 nuclease digestion. A gel containing 25 µg of sample DNA in each lane was run on a 1% agarose gel and stained with ethidium bromide. The gel was scanned with a PhosphorImager (Molecular Dynamics). Nuclease digestion of genomic DNA was performed using 25 units of micrococcal nuclease (New England Biolabs, Beverly, MA) for 15 min at 37 °C. The digestion mixture was then treated with 5% SDS and extracted with phenol/chloroform. The digest was ethanol precipitated and resuspended in TE to a final concentration of 2 µg/µl. This DNA was used as a control for S1 nuclease digestion. A gel containing 25 µg of sample DNA in each lane was run on a 1% agarose gel and stained with ethidium bromide. The gel was scanned with a PhosphorImager (Molecular Dynamics). Nuclease digestion of genomic DNA was performed using 25 units of micrococcal nuclease (New England Biolabs, Beverly, MA) for 15 min at 37 °C. The digestion mixture was then treated with 5% SDS and extracted with phenol/chloroform. The digest was ethanol precipitated and resuspended in TE to a final concentration of 2 µg/µl. This DNA was used as a control for S1 nuclease digestion. A gel containing 25 µg of sample DNA in each lane was run on a 1% agarose gel and stained with ethidium bromide. The gel was scanned with a PhosphorImager (Molecular Dynamics). Nuclease digestion of genomic DNA was performed using 25 units of micrococcal nuclease (New England Biolabs, Beverly, MA) for 15 min at 37 °C. The digestion mixture was then treated with 5% SDS and extracted with phenol/chloroform. The digest was ethanol precipitated and resuspended in TE to a final concentra...
was identified 363 bp downstream from the ATG translation start site, where the genomic sequence diverged from the PKC-\(\eta\) cDNA sequence (Fig. 2). The first exon encodes 121 amino acids (Fig. 2). Further sequencing of exon 1 in the 3’ direction yielded no cDNA sequences. It follows that the second exon was separated by at least 4.7 kb intronic sequences.

Chromosomal Localization of the PKC-\(\eta\) Gene—Chromosomal fluoroscence in situ hybridization using PKC-\(\eta\) P1 clone 7239 as probe resulted in specific labeling of the long arm of a group D chromosome (Fig. 3). This chromosome was tentatively identified as number 14 based on DAPI staining. This identification was confirmed by double staining with the P1 clone probe and a biotin-labeled probe that was specific for the centromeres of chromosomes 14 and 22. This double staining resulted in specific labeling of the centromere of chromosome 14 in red and the long arm in green (Fig. 3). Based on ten independent measurements, the PKC-\(\eta\) hybridization signal was located 47% of the distance from the centromere to the telomere of chromosome arm 14q, an area that corresponds to the interface between bands 14q22–23.

Identification of Transcription Start Site—As stated above, the proximal promoter region of the PKC-\(\eta\) gene lacked sequences indicative of transcription initiation sites. For this reason, several complementary approaches were taken to determine the transcription start site. The first approach utilized RT-PCR to delineate the region of transcription initiation. First-strand cDNA was synthesized from HaCaT cell total RNA using antisense primer \(\Pi\) (Fig. 4), which hybridized 45 bp downstream of the translation initiation site. This first strand cDNA was then used as a template for PCR amplification with six primer pairs consisting of a common antisense primer \(\Pi\) (Fig. 4) and a series of sense primers located 334 bp to 974 bp upstream from \(\Pi\) (Fig. 4). PCR amplification using the same six primer pairs with cloned PKC-\(\eta\) genomic DNA as template served as positive control. PCR products of the expected sizes were observed with all six primer pairs using PKC-\(\eta\) DNA as template (Fig. 4). RT-PCR products of the expected sizes were also observed with primer pairs G/F (334 bp), G/E (484 bp), and G/D (588 bp) (Fig. 4). In contrast, no RT-PCR products were found with primer pairs G/C (705 bp), G/B (799 bp), or G/A (974 bp) (Fig. 4), indicating that genomic sequences contained within primers A/C were not present in the PKC-\(\eta\) cDNA. These data indicate that the 5’ end of the PKC-\(\eta\) transcript lies within the 93-bp region between primers D and C.

We next employed primer extension analysis to narrow down the location of the transcription initiation site. Antisense primer I (Fig. 5A), which annealed 279 bp downstream of primer \(\Pi\) (Fig. 4), was end-labeled and annealed to HaCaT cell total RNA and extended by Superscript II reverse transcriptase. Two major primer extension products were observed, approximately 260 bp in length (Fig. 5B, lane 3). No extended products were observed with yeast tRNA as template, a control for the specificity of primer hybridization (Fig. 5B, lane 2). Sequence analysis revealed that the two alternative transcription start sites were separated by 12 nucleotides (Fig. 5C).

To confirm the primer extension results, we performed S1 nuclease mapping using a probe, generated with antisense primer J (Fig. 3A) and sense primer C (Fig. 4), that extended 26 nucleotides upstream of the distal transcription start site identified by primer extension analysis. Two major S1 nuclease-protected fragments of 431 and 418 nucleotides were observed with HaCaT cell total RNA as template (Fig. 5D, lane 4). The sizes of these two protected fragments exactly matched the expected transcription start sites identified by primer extension. No protected fragments were observed using yeast tRNA as template (Fig. 5D, lane 3). Taken together, the above RT-
PCR, primer extension, and S1 nuclease mapping data demonstrate that the transcription initiation site of the human PKC-\(\eta\) gene is located approximately 650 bp upstream from the ATG translation initiation site.

Cell-specific Regulation of the 5'-Upstream Region of the PKC-\(\eta\) Gene—In human skin in vivo, PKC-\(\eta\) is expressed in...
epidermal keratinocytes but not in dermal fibroblasts (5). RT-PCR detected PKC-\(\eta\) transcripts in human skin, primary cultured human skin keratinocytes, and human keratinocyte HaCaT cells but not in human skin fibroblasts (Fig. 6A). We therefore examined whether the 5'-flanking region of the PKC-\(\eta\) gene also displayed cell type-specific regulation. The full-length 5'-flanking sequence of the PKC-\(\eta\) gene (−3458 to +708) was inserted into the promoterless pCAT3-Basic reporter gene and transiently transfected into primary human keratinocytes, human keratinocyte HaCaT cells, and human skin fibroblasts. Transient transfection of the pCAT3-Control reporter plasmid (containing the SV40 promoter and SV40 enhancer) was used as positive control. The promoterless pCAT3-Basic reporter gene was unable to drive expression of CAT activity, whereas the pCAT3-Control reporter gene expressed high levels of CAT activity in each of the three cell types (Fig. 6B). The PKC-\(\eta\) gene reporter construct displayed significant activity in keratinocytes and HaCaT cells (at least 10-fold higher than the promoterless CAT expression vector) but was inactive in fibroblasts (Fig. 6B). These data demonstrate that the upstream region of the PKC-\(\eta\) gene contains regulatory elements sufficient to drive cell type-specific transcription.

Functional Analysis of the 5'-Flanking Region of the PKC-\(\eta\) Gene—To identify cis-acting regulatory elements in the 5'-flanking region of the PKC-\(\eta\) gene, we constructed a series of 5' deletion CAT expression vectors and transiently transfected them into primary keratinocytes and keratinocyte HaCaT cells (Fig. 7). The promoter activities of the various constructs obtained from 6 to 11 independent experiments in HaCaT cells are summarized in Fig. 7. Similar data were obtained for primary keratinocytes (data not shown). All CAT constructs were also expressed in human skin fibroblasts but were found to be inactive (data not shown).

As described above (Fig. 6B), the largest construct, a 4.1-kb fragment extending from −3458 to +708 (Fig. 7, construct 83CN), displayed significant CAT activity. The activity of this construct was assigned a relative level of 1.0. 5' deletion of 1251 bp (Fig. 7, construct 82CN) did not significantly alter CAT activity. However, further 5' deletion of 566 bp (Fig. 7, construct 87CN) resulted in a 10-fold increase in CAT activity, suggesting the presence of a silencer-like element(s) between −2207 bp and −1641 bp. Additional 5' deletion of 442 bp (Fig. 7, construct 89CN) decreased CAT activity approximately 50%. Further 5' truncation of 115 bp (Fig. 7, construct 78C) reduced CAT activity to its initial relative value of 1.0, suggesting that the region between −1641 and −1084 bp contains an enhancer-like element(s). Further 5' deletion (Fig. 7, construct 78C-75C) essentially abolished CAT activity.

We tested two constructs, −1641 to −611 (Fig. 7, construct 124C) and −1199 to −611 (Fig. 7, construct 125C), to determine whether the enhancer-like region identified between −1641 and −1084 bp could function independently of the PKC-\(\eta\) prox-
Next, we further characterized the gous Promoter Activity—

...cloned upstream of the minimal thymidine kinase promoter. The PKC-\(\eta\) gene transcription.

Human PKC-\(\eta\) Gene Promoter

Characterization of Enhancer Sequences—To more precisely identify enhancer sequences, we analyzed a series of 5’ deletion constructs within the enhancer region (−1641 to −1079) for enhancer activity in HaCaT cells. 5’ deletion of 355 bp increased enhancer activity 30% (Fig. 9, constructs 96CNN through 130P). 5’ deletion of an additional 27 bp reduced enhancer activity to its initial level (Fig. 9A, construct 131P). Deletion of an additional 70 bp abolished enhancer activity (Fig. 9A, construct 101P). These data indicate that enhancer sequences lie between −1259 and −1189 of the PKC-\(\eta\) distal promoter.

Characterization of Silencer Sequences—To more precisely identify sequences within the silencer region (−2207 to −1641), human keratinocytes, and human skin fibroblasts. In HaCaT cells, the silencer region reduced pBLCAT2 activity by 80% and pCAT3-promoter activity by 70% (Fig. 8). The enhancer region elevated both pBLCAT2 activity (3.5-fold) and pCAT3-promoter activity (4-fold). Similar results were obtained in cultured human keratinocytes (data not shown). In contrast, neither the silencer nor the enhancer regions had any effect on reporter gene activity in skin fibroblasts (data not shown).

PKC-\(\eta\) Silencer and Enhancer Regions Regulate Heterologous Promoter Activity—Next, we further characterized the putative silencer-like region (−2207 to −1641) and enhancer-like region (−1641 to −1084) in the 5’-flanking region of the PKC-\(\eta\) gene. The enhancer and silencer sequences were separately cloned upstream of the minimal thymidine kinase promoter in the CAT reporter plasmid pBLCAT2, and the SV40 promoter CAT reporter plasmid pCAT3-promoter. The constructs were then transiently transfected into HaCaT cells, human keratinocytes, and human skin fibroblasts. In HaCaT cells, the silencer region reduced pBLCAT2 activity by 80% and pCAT3-promoter activity by 70% (Fig. 8). The enhancer region elevated both pBLCAT2 activity (3.5-fold) and pCAT3-promoter activity (4-fold). Similar results were obtained in cultured human keratinocytes (data not shown). In contrast, neither the silencer nor the enhancer regions had any effect on reporter gene activity in skin fibroblasts (data not shown).

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Computer analysis of enhancer sequences identified a GA-rich region that contained three GGA repeats that constituted overlapping potential binding sites for Ets (−1233/−1226) and HSF (−1243/−1220) transcription factors. EMSA, using a probe spanning the GA-rich region (−1244/−1221) revealed specific protein-DNA complexes with HaCaT cell nuclear extract (Fig. 9B, lanes 1–4). In addition, competition experiments with excess unlabeled probes containing consensus binding sites for Ets or HSF transcription factors effectively reduced binding of HaCaT cell nuclear proteins to the PKC-\(\eta\) GA-rich region (−1244/−1221) probe (data not shown). Incubation of nuclear extracts with antibody to Ets 1/2 (Fig. 9B, lanes 5 and 6) or HSF-1 (Fig. 9B, lanes 7 and 8) yielded weak supershifted complexes.

To test whether the three GGA repeats contribute to enhancer activity, each GGA within the enhancer region (−1286 to −1079) was mutated, and the effect of mutation on enhancer activity was determined in HaCaT cells. Mutation of one GGA reduced enhancer activity 20%, whereas mutation of two or three GGA sequences revealed a similar pattern in EMSA. Mutation of one GGA sequence had minimal effect on formation of DNA-protein complexes, whereas mutation of two or three GGA sequences substantially reduced the intensity of specific retarded complexes (data not shown).

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we analyzed a series of 5’ deletion CAT constructs for silencer activity in HaCaT cells. The silencer region inhibited promoter activity approximately 50% (Fig. 10A, construct 114B). Deletions of 262 bp did not alter silencer activity (Fig. 10A, constructs 116B and 118B). 5’ deletion of an additional 124 bp resulted in complete inhibition of thymidine kinase promoter activity (Fig. 10A, construct 120B). Further 5’ deletion of 119 bp abolished all silencer activity (Fig. 10A, construct 473). These data indicate that silencer region sequences lie between -21821 and -21702 of the PKC-η distal promoter.

Computer analysis of the silencer sequences (-1821 to -1702) identified AP-1 (-1740 to -1734) and Ets (-1757 to -1754 and -1715 to -1712) consensus binding sites. EMSA, using a probe containing the AP-1 and Ets elements (-1772 to -1703), revealed specific protein-DNA complexes with HaCaT cell nuclear extract (Fig. 10B, lanes 1–4). However, incubation of nuclear extracts with antibody to c-Jun, c-Fos, or Ets 1/2 individually or in combination (Fig. 10B, lanes 5–10) did not reveal any supershifted complexes. In addition, excess unlabeled probes containing consensus binding sequences for AP-1 (c-Jun/c-Fos) or Ets did not compete for binding of HaCaT nuclear extract proteins to the silencer probe (data not shown). These data suggest that neither AP-1 nor Ets proteins bind to the PKC-η silencer element.

**DISCUSSION**

PKC-η expression is highly tissue- and cell type-specific: it is expressed predominantly in epithelial tissues such as skin,
to test the tissue specificity of PKC-\(\eta\) gene expression, we introduced a series of PKC-\(\eta\) promoter/CAT reporter gene constructs into human keratinocyte HaCaT cells, primary human skin keratinocytes, and human skin fibroblasts. Our results clearly demonstrate that the full-length (3.6 kb) 5'-flanking sequence of the PKC-\(\eta\) gene contains functional promoter and cis-elements that are capable of conferring keratinocyte-specific expression of the PKC-\(\eta\) gene. The promoter CAT constructs were not active in skin fibroblasts, consistent with a lack of endogenous PKC-\(\eta\) mRNA expression in these cells.

Deletion analysis of PKC-\(\eta\) promoter/CAT constructs revealed the presence of silencer and enhancer elements in the distal 5'-upstream region. The silencer region repressed (80%), and the enhancer region stimulated (4-fold) basal activity of two heterologous promoters when transfected into human keratinocyte HaCaT cells or primary human keratinocytes. In contrast, neither the silencer nor the enhancer were active in skin fibroblasts. These data suggest that the silencer and enhancer elements may play an important role in keratinocyte-specific expression of the PKC-\(\eta\) gene.

5' deletion analysis narrowed down enhancer sequences to 70 bp lying between −1259 and −1189. This region is GA-rich, containing overlapping consensus binding sites for Ets and HSF transcription factors. Mutation of these binding sites resulted in loss of DNA binding and enhancer activity. Mutation of two sites resulted in greater loss of DNA binding and enhancer activity than mutation of one site. These data suggest that both binding sites are required to form stable DNA complexes. Both Ets and HSF members bind DNA as dimeric or trimeric complexes, respectively (31). Unlabeled consensus Ets and HSF probes effectively competed for binding of HaCaT cell nuclear proteins to enhancer sequences. However, antibodies to Ets 1/2 and HSF-1 yielded only weak supershifted bands, suggesting that other proteins are present in the retarded complexes.

Deletion analysis narrowed down silencer activity to 119 bp between −1821 and −1702 of the PKC-\(\eta\) promoter. These sequences completely inhibited activity of the heterologous thymidine kinase promoter. Consensus AP-1 (−1740 to −1734) and Ets (−1257 to −1254 and −1215 to −1212) transcription factor binding sites were located within the silencer, and a DNA probe containing the AP-1 and Ets elements formed specific complexes with HaCaT cell nuclear proteins. These data are consistent with binding of AP-1 and Ets family members to have been partially characterized, also have large gaps between their first and second exons (25, 26). The PKC-\(\eta\) gene contains two major transcription initiation sites, which are located approximately 650 bp upstream from the ATG translation initiation site. The 3.6-kb 5'-flanking region of the PKC-\(\eta\) gene lacked canonical TATA and CAAT boxes adjacent to the transcription start sites. Both PKC-\(\gamma\) and PKC-\(\beta\) also lack TATA and CAAT boxes (25, 26). These features are consistent with identification of multiple transcription initiation sites in a TATA-less promoter region. Many genes that lack TATA and CAAT boxes at the usual positions have multiple transcription initiation sites (27–30). TATA and CAAT elements, in reverse order, were found further upstream at −3448, −3428, and −2677, respectively. However, we could not detect any transcription in the vicinity of these TATA or CAAT boxes employing either primer extension, S1 nuclease protection, or RT-PCR. Moreover, no significant transcriptional activity in CAT constructs was observed in this region, suggesting that the TATA and CAAT elements were not functional. The proximal promoter region for PKC-\(\eta\) is not GC-rich. However, the 5'-untranslated region is extremely rich in GC (80%).

To examine the functionality of the PKC-\(\eta\) promoter and to test the tissue specificity of PKC-\(\eta\) gene expression, we introduced a series of PKC-\(\eta\) promoter/CAT reporter gene constructs into human keratinocyte HaCaT cells, primary human skin keratinocytes, and human skin fibroblasts. Our results clearly demonstrate that the full-length (3.6 kb) 5'-flanking sequence of the PKC-\(\eta\) gene contains functional promoter and cis-elements that are capable of conferring keratinocyte-specific expression of the PKC-\(\eta\) gene. The promoter CAT constructs were not active in skin fibroblasts, consistent with a lack of endogenous PKC-\(\eta\) mRNA expression in these cells.

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Deletion analysis narrowed down silencer activity to 119 bp between −1821 and −1702 of the PKC-\(\eta\) promoter. These sequences completely inhibited activity of the heterologous thymidine kinase promoter. Consensus AP-1 (−1740 to −1734) and Ets (−1257 to −1254 and −1215 to −1212) transcription factor binding sites were located within the silencer, and a DNA probe containing the AP-1 and Ets elements formed specific complexes with HaCaT cell nuclear proteins. These data are consistent with binding of AP-1 and Ets family members to
silencer sequences.

3' deletion of sequences between the transcription initiation site and the enhancer region in the PKC-δ gene promoter abolished enhancer activity, indicating that the proximal promoter was essential for enhancer function. The proximal promoter alone (i.e., without the enhancer), however, could not drive transcription. The proximal promoter contains several potential Sp1 and Ets motifs. Both Sp1 and Ets transcription factor families are expressed in a variety of cell types (20, 32), including human keratinocytes (33, 34). Sp1 and Ets are known to function in conjunction with other transcription factors to positively regulate several differentiation-related genes in keratinocytes (35). Interestingly, skin SPRR2A (36) and involucrin (37) genes, which are involved in keratinocytes (34). Sp1 and Ets are also essential for expression of SPRR2A (36) and involucrin (37) genes, which are involved in keratinocyte terminal differentiation (36). Interestingly, skin expresses a novel Ets family member named Jen (38) or ESE-1 (39), whose expression coincides with that of PKC-δ, i.e., restricted to keratinocytes undergoing the later stages of differentiation. Sp1 and Ets members, along with other transcription factors such as AP-1 and AP-2, may coordinately regulate expression of PKC-δ and other genes involved in keratinocyte differentiation (35).

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