Journal of Cellular Biochemistry 73:355–369 (1999)

Transcription Regulatory Elements of the First Intron Control Human Transglutaminase Type I Gene Expression in Epidermal Keratinocytes

Renata R. Polakowska,[1]* B.A. Graf,[2] V. Falciano,[3] and P. LaCelle[1]

[1]University of Rochester Medical Center, Department of Dermatology, Rochester, New York 14642
[2]University of Rochester Cancer Center, Rochester, New York 14642
[3]University of Rochester Medical Center, Rochester, New York 14642

Abstract Expression of the transglutaminase type1 gene (TGM1), which encodes an epithelial cell-specific protein cross-linking enzyme, is limited to particular stages of epidermal development and keratinocyte differentiation. As a result, transglutaminase type 1 (TGase1) enzyme activity in epidermal cells increases with the onset of keratinization in vivo and in vitro. We determined, by functional mapping of deletion mutations in the TGM1 5′ untranslated region, that an element in first intron of the human TGM1 gene, in addition to the 5′ proximal promoter, initiates transcription and upregulates transcriptional activity. These two transcription control elements function interdependently to regulate the expression of the human TGM1 gene in keratinocytes. We also identified distinct regulatory elements that cooperatively modify the 5′ proximal and intron 1 promoter activities in response to environmental variations in retinoic acid and calcium ion concentrations. In conclusion, we report that TGM1 differential gene expression is controlled by two distinct elements, proximal and intronic, which function cooperatively to initiate and modulate TGM1 gene transcription in response to regulatory signals. We propose that in nonexpressing cells these regulatory signals repress a default mechanism that operates in their absence. The specificity of their function is integrated into the default mechanism and consists of the tissue-, developmental-, and differentiation-specific interplay of 5′ URR and intron 1 elements tuned to physiological status. J. Cell. Biochem. 73:355–369, 1999. © 1999 Wiley-Liss, Inc.

Key words: epidermal keratinocytes; TGM1; gene regulation; transglutaminase, intron 1

Transglutaminases catalyze post-translational modification of proteins, forming e-(γ-glutamyl) lysine isopeptide cross-links between precursor proteins and polyamines, creating insoluble polymerized structures with diverse biological functions [Polakowska and Goldsmith, 1991; Lorand and Conrad, 1984; Folk, 1996; Aeschlimann and Paulsson, 1994; Greenberg et al., 1991]. TGases have been identified in all organisms studied, from bacteria to mammals [Aeschlimann and Paulsson, 1994; Rice et al., 1992]. Despite their similar catalytic activities and homologous primary structures, the growing number of known TGases can be distinguished on the basis of biological function, substrate specificity, immunoreactivity, gene organization and regulation [Reichert et al., 1993; Greenberg et al., 1991; Parenteau et al., 1986], and tissue distribution.

Transglutaminase type I (TGase1), or keratinocyte transglutaminase (TGase K), protein cross-linking plays a major role in the assembly of the insoluble cornified cell envelope, a hallmark of terminally differentiated keratinocytes that contributes to the structural stability and barrier function of the epidermis [Polakowska and Goldsmith, 1991; Roop, 1995; Hohl, 1990]. Defects in the transglutaminase type 1 (TGM1) gene interfere with normal cornified cell envelope formation resulting in the severe skin disorder lamellar ichthyosis (LI) [Huber et al., 1995]. Homozygous TGM1-/- knockout mice are born with an abnormal epidermis symptomatic of the human disease, and die soon after birth [Matsuki et al., 1998]. Ectopic expression of TGM1 in primary lamellar ichthyosis keratinocytes restored some features of the normal phenotype, providing a basis for gene therapy in treatment of this and other skin disorders [Cho-

*Correspondence to: Renata R. Polakowska, University of Rochester Medical Center, Box 697, 601 Elmwood Ave, Rochester, NY 14642. E-mail: Renata_Polakowska@urmc.rochester.edu

Received 24 September 1998; Accepted 25 November 1998

© 1999 Wiley-Liss, Inc.

ate and Khavari, 1997]. Genetic heterogeneity has been revealed through analysis of families with LI, and at least five TGM1 mutations have been identified in association with this disease [Hennies et al., 1998]. Further, heterogeneity in the TGase family has been identified with the recent isolation of the TGx cDNA, the fourth form of transglutaminase expressed in keratinocytes [Aeschlimann et al., 1998].

Progression of epidermal maturation from basal cells to dead cells of the stratum corneum is accompanied by differential expression of the TGases [Kim et al., 1994; Polakowska et al., 1994; Eckert et al., 1997b; Thacher and Rice, 1985]. Undifferentiated basal cells in the native epidermis and basal-like keratinocytes cultured in low Ca^{2+} medium express predominantly the ubiquitous tissue transglutaminase, or TGase2, which functions in the formation of apoptotic bodies [Haake and Polakowska, 1993; Fesus et al., 1991] and in cellular signaling [Nakaoka et al., 1994]. The TGase1 enzyme comprises by far the majority of TGase protein in differentiating cells, and is limited to the spinous and granular layers of stratifying epithelium in vivo and to keratinocytes induced to differentiate in vitro. The TGase3 enzyme appears even later in differentiation, restricted to keratinocytes of the upper granular layer and to inner root sheath cells of hair follicles [Lee et al., 1996]; the gene is also transcriptionally active in cultured keratinocytes [Lee et al., 1996; Kim et al., 1994]. These three TGases also have distinct patterns of expression during fetal skin development [Polakowska et al., 1994], suggesting the need for tight regulation and coordinated expression for proper keratinocyte growth and differentiation.

The precise molecular mechanisms that control expression of these three TGase genes during keratinocyte differentiation are still poorly understood. Steady-state TGM1 mRNA levels increase with the advance of terminal differentiation [Floyd and Jetten, 1989; Gibson et al., 1996; Dlugosz and Yuspa, 1994]. Retinoic acid (RA) suppresses both keratinocyte terminal differentiation [Fisher and Voorhees, 1996; Fuchs, 1990] and TGM1 mRNA expression in vitro [Yamada et al., 1994; Vollberg et al., 1992; Saunders et al., 1993]. In vivo, however, RA induces expression of the TGM1 gene [Griffiths et al., 1992] implying more complicated regulatory mechanisms distinguished by differences in the in vivo and in vitro growth conditions. Importantly, the coregulation of TGM1 gene expression and keratinocyte differentiation suggests that the mechanisms underlying TGM1 gene expression may pertain to the regulation of terminal differentiation generally.

To elucidate the molecular mechanisms involved in this regulation, we isolated and characterized genomic clones encoding TGM1 [Polakowska et al., 1992]. In the present study we analyzed in greater detail the K3 subclone containing the 5′ untranslated region (5′ UTR) of the human TGM1 gene, consisting of untranscribed sequences upstream of the untranslated exon I, exon I and the first intron. In similar studies which involved functional mapping of the rabbit TGM1 gene, a sequence element required for TGM1 core promoter activity was identified within 84 bp of the transcription initiation site [Saunders et al., 1993]. Mapping of the human TGM1 upstream regulatory region (URR) in rat cells identified a core promoter within 95 nucleotides of the CAP transcription initiation site [Ueda et al., 1996]. The human TGM1 region of 2,200 bp proximal to the CAP site was essentially inactive in human SCC-9 or SIK keratinocyte cell lines compared to rat cells [Mariniello et al., 1995], indicating that the promoter upstream of the transcription start site is either insufficient or suppressed in those cells. Both the rabbit and human TGM1 URR are responsive to TPA and RA [Yamada et al., 1994; Saunders et al., 1993], and human TGM1 promoter activity is also modulated by overexpression of Jun and Fos proteins of the AP1 family [Yamada et al., 1994].

We show that the TGM1 gene expression is upregulated by elements within intron 1 and demonstrate that transcription of the human TGM1 gene is controlled by the cooperative function of intronic and proximal control elements, which include the previously described proximal promoter located upstream of exon 1 and a promoter within the first intron, introduced in this study. The two promoters are regulated by RA- and Ca^{2+}-responsive elements within separate regulatory regions, and overall gene activity is controlled in a tissue-, developmental-, and differentiation-specific manner by the interplay of their respective regulatory sequences.

MATERIALS AND METHODS

Cell Culture

The HaCaT cell line of spontaneously immortalized human keratinocytes was obtained from Drs. N. Fusenig and D. Breitkreutz and cultured as recommended [Boukamp et al., 1988]. Neonatal Human Keratinocyte (NHK) cultures were initiated in Keratinocyte Growth Medium (KGM) (Clonetics, San Diego, CA) from epidermal cell suspensions obtained by trypsin dissociation of dispase-separated epidermis. Keratinocytes were initially cultured in low calcium KGM (0.1 mM Ca^{2+}) and passaged at approximately 80% confluence. First or second passage cells were induced to differentiate by switching to high calcium KGM (1.2 mM Ca^{2+}). Fibroblasts were initiated in RPMI+10% fetal calf serum after collagenase digestion of isolated dermis.

Constructs with Deletion Mutations

Deletion mutations were introduced by linking various portions of the TGM1 5′-flanking region (-929 to +822) to the CAT reporter gene in the promoterless pBLCAT3 [Luckow and Schutz, 1987] or pCAT-basic (Promega, Madison, WI) vectors. The 194 bp NlaIII DNA fragment (+628 to +822) of the TGM1 5′ upstream region, immediately upstream of the AUG codon, was ligated into the SphI site of the promoterless vectors to construct the p194 chimeric plasmid. The pH and pB plasmids were constructed by insertion of the HindIII and BamHI DNA fragments into compatible sites in the parent vectors, respectively. The p194H plasmid was constructed by digestion of the p194 plasmid with HindIII and insertion of the TGM1 HindIII fragment. This plasmid served as the base for constructing the pK3 plasmid which consisted of the entire sequence 5′ of exon II of the genomic EMBL-K clone isolated previously [Polakowska et al., 1992]. The deletion mutated construct p194HΔ was constructed by removal of the TGM1 PstI fragment from p194H parent plasmid.

The p3.0(+)int, or intron plus, construct contains the full length regulatory region including intron 1 (a 3.0 kb SacI/NaeIII genomic DNA fragment proximal to the first AUG codon of the TGM1 gene which includes 2170 bp 5′ upstream of the CAP site, 94 bp of exon I and 737 bp of the TGM1 first intron), while the p3.0(-)int, or intronless, construct does not contain the intron 1 sequences nor 27 nucleotides at the 3' end of exon I up to the Sca I restriction site. Both intron plus and intronless DNA fragments were inserted into the pSPCAT vector in which the 5′ T7 RNA polymerase promoter was removed.

Liposome-Mediated Transfection

Plasmid DNA was introduced into keratinocytes and fibroblasts by lipid-mediated transfection. Liposomes were prepared according to Rose et al. [1991] with 5 mg/ml of the cationic lipid dimethyldioctadecylammonium bromide (DDAB), and 1 mg/ml of the neutral lipid dioleoyl-L-α-phosphatidylethanolamine (PtdEtn). Keratinocytes and fibroblasts grown in 60 mm culture dishes were treated at 50–70% confluence with a transfection mixture of 1.5 ml Dulbecco's modified Eagle medium (DMEM),12 μg of reporter plasmid DNA supplemented with a standard amount of a β-galactosidase expression vector, and 45 μl of the lipid mix. After 4h the dishes were supplemented with an equal volume of KGM, or RPMI 1640 with 4% serum, for an additional 2h, followed by replacement of the media with KGM or RPMI/2% serum with or without high (1.2 mM) Ca^{2+} and RA (10^{-6} M) for 48h. Cell lysates were analyzed for transcriptional activity by CAT assay and for transfection efficiency by β-galactosidase assay. Data from five experiments revealed the same trends; variation in the magnitude of CAT levels between experiments undermines the usefulness of statistical comparison of means. Statistical analysis of variance indicated with high confidence a difference between all constructs for NHK and HaCaT cells.

Primer Extension and RT-PCR

A 30 bp oligonucleotide complementary to the CAT gene mRNA was [γ-^{32}P] ATP phosphorylated and hybridized to 5 μg of the poly (A)+ RNA isolated from transiently transfected keratinocytes for 3h at 60°C in 10 mM Tris (pH 8.3), 1 mM EDTA, 250 mM KCl annealing buffer. Reverse transcription was performed in 16.7 mM KCl. 13.3 mM $MgCl_2$, 23.3 mM Tris (pH 8.3), 13.3 mM dithiothreitol (DTT), 0.33 mM dNTP, and 0.133 mg/ml actinomycin D with 200 units of reverse transcriptase. The extension products were fractionated on a 6% sequencing gel and autoradiographed.

For RT-PCR the following primers were used: intron 1 - #307 5′CACCCTGCCTCTTCCTAAG AG3’ and #547 5′GGAGCACTCTGATGTGT GTG3’; exon 1 - #281 5′TCTGTGGGTCCTGTC CCATCCA3’; exon 2 - #159 5′CCCCAACGGCC CACATCGGAACGTGGCCCATCCATCATGC3’; pUC19- #104 5′ACGACGGCCAGTGCCA AGC3’; CAT - #490 5′TTAGCTTCCTTAGCTCC TGA-AAATCTCGCC3’. The cDNA Cycle kit (Invitrogen, La Jolla, CA) was used to reverse transcribe cDNA from total or poly(A) RNA isolated from neonatal keratinocytes cultured in the presence of high Ca^{2+} and from neonatal fibroblasts for control, followed by amplification of specific cDNA by PCR. The human β-actin cDNA was PCR amplified to control for the reverse transcription reaction.

Gel Retardation Assay

Ten μg of each nuclear protein extracts from keratinocytes grown in high Ca^{2+} and low Ca^{2+} medium, prepared according to the procedure of Dignam et al. [1983] were incubated with the [^{32}P]-labeled 194 bp NlaIII DNA fragment from the TGM1 first intron region for 15 min at 30°C (20 mM HEPES pH7.5, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 100 mM KCl) with 2 μg poly(dIdC) to decrease nonspecific binding. Protein concentration was established by the Bradford method using a BSA standard curve. DNA-protein complexes were separated by 4% polyacrylamide gel electrophoresis and detected by autoradiography.

RNase Protection Assay

The RNase protection assay was performed using the RPA II kit (Ambion) according to the manufacture’s recommendations. Sp6 polymerase was used to synthesize the 194 bp NlaIII fragment of the TGM1 first intron as the antisense RNA probe. A β-actin RNA probe was also hybridized serving as a control. Unprotected RNA was also RNase-digested as a control. The pCDNA1 vector (Promega) containing this fragment was linearized with EcoRI to terminate synthesis of the RNA. Sequencing reactions performed on an irrelevant template served as size standards. Following sequencing polyacrylamide gel electrophoresis, protected bands were detected by autoradiography.

RESULTS

Transcriptional Activity of the TGM1 Gene is Enhanced by Regulatory Elements of the First Intron

Human TGase1 is an 817 amino acid protein encoded by a single gene, TGM1, located on chromosome 14q.11.2 [Yamanishi et al., 1992; Kim et al., 1992; Polakowska et al., 1991]. The transcription unit of the gene consists of 15 exons and 14 introns. We show here, as others have shown [Phillips et al., 1992; Yamanishi et al., 1992; Kim et al., 1992], that the exon I size is variable, perhaps reflecting multiple transcription start sites. The +1 CAP site mapping to the “G” nucleotide 94 bp upstream of the 5′ exon/intron splice site was determined by extension of the #159 primer complementary to 30 nucleotides at the 5′ end of exon II (data not shown). The transcription start site at the “G” nucleotide was in agreement with one of the sites mapped by Yamanishi et al. [1992], and we propose that it establishes the size of exon I as 94 bp.

To identify the functional *cis*-acting regulatory sequences responsible for differential expression of the TGM1 gene during skin differentiation and development, 3.0 kb of the 5′ region upstream of the ATG translation initiation codon were inserted into promoterless CAT reporter vectors. The p3.0(-)int, or intronless reporter construct, lacking intron 1 and 27 nucleotides at the 3’ end of exon I, expressed low levels of the CAT reporter gene in transfected neonatal human keratinoyctes (NHK). Restoring intron 1 in the p3.0(+)int, or intron plus, construct increased expression of the CAT reporter gene 10 times (Fig. 1), a level 134-fold higher than the promoterless vector, indicating that the 3.0 kb untranslated sequence of the TGM1 gene is sufficient to promote reporter and presumably human TGM1 gene transcription and that important transcription regulatory elements lie within intron 1. In fibroblasts, neither intronless nor intron-containing constructs directed CAT expression, demonstrating that the intron 1 enhancing activity is cell type-specific and that the 5′ URR contains sufficient information for the keratinocyte-specific expression of the TGM1 gene. We next performed functional mapping of the 5′ end region of the gene to further detail the molecular mechanisms that operate to control TGM1 expression in these cells.

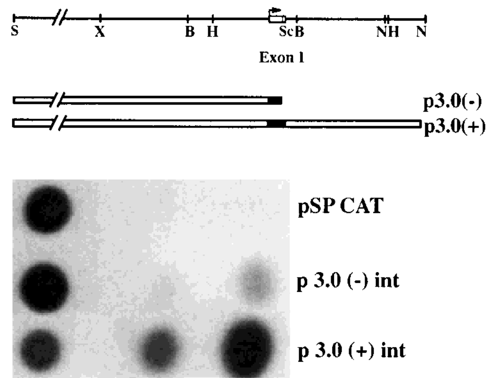

Fig. 1. CAT activity driven by the intron-plus and intronless TGM1 5′-untranslated region assessed in undifferentiated neonatal human keratinocytes (NHK). Transfected keratinocytes were harvested 48h post transfection. Upper line shows restriction map of the TGM1 5′-untranslated region starting upstream of the ATG translation initiation codon; upper case letters represent restriction enzyme sites: S, SacI; X, XhoI; B, BamHI; H, HindIII; N, NlaIII. Middle open bars represent intronless p3.0 (-)int and intron-plus p3.0(+)int constructs; closed box = exon I. At bottom is shown representative CAT assay results; a promoterless pSPCAT plasmid served as a negative control.

Intron 1 and the 5′ Upstream Regulatory Region of TGM1 Contain Interdependent Control Elements

To delineate elements with enhancing activity, the previously described 1753 bp K3 region at the 5′ end of the human TGM1 gene (from +830 to -923) [Polakowska et al., 1992], which contains 923 bp of flanking region 5′ of the CAP site, 94bp of exon I, and the 736 bp first intron (Fig. 2A), was studied in more detail. Neonatal human keratinocytes (NHK) grown in low Ca^{2+} (0.1 mM) medium and spontaneously immortalized HaCaT keratinocytes were transfected with a series of reporter constructs containing K3 deletion mutations (Fig. 2B), and lysates were anaylzed for CAT activity.

In low Ca^{2+} medium which maintains the primary cells in a proliferative and undifferentiated state, which is also characteristic of HaCaT cells, the pK3 construct directed strong CAT gene expression in NHK and to a lesser extent in HaCaT cells (Fig. 3). Deletion of the 5′ XhoI/HindIII DNA fragment (p194H) reduced the CAT activity in NHK by 80%, identifying the XhoI/HindIII or 5′ X/H fragment as a positive control element. Interestingly, deletion of this element had little effect on CAT activity in HaCaT cells, implying that the 5′ X/H element is inactive in these cells.

The 5′ X/H element present in p3.0(-)int was, however, insufficient for maximal expression of the CAT reporter gene (Fig. 1). Maximal expression was dependent on the presence of both the 5′ X/H proximal element and intron 1 of the TGM1 gene. Deletion most of both, intron 1 and the 5′X/H elements, but preserving the 5′ sequences of the proximal core promoter (pB; Fig. 2A,B), reduced the CAT activity by 90% relative to the intact pK3 in NHK (Fig. 3). In contrast, pB produced a higher CAT activity than the full length pK3 in HaCaT (Fig. 3), underlining the cell type-specificity of TGM1 gene regulation. The pH deletion construct (Fig. 2B) lacking 194 nucleotides at the 3′end of intron 1 was nearly inactive in both HaCaT and normal keratinocytes (Fig. 3), suggesting that the 194 nucleotides of intron 1 are important for efficient TGM1 gene expression. Surprisingly, the 194 bp fragment alone (p194, Fig. 2B) was able to drive CAT expression (Fig. 3) and at reproducibly higher levels than p194H which contains the proximal promoter, particularly in HaCaT cells (Fig. 3). This identifies the 194 bp region of intron 1 as a promoter of CAT transcription, and potentially as a second promoter of TGM1 gene expression in human keratinocytes. It was of interest next to determine whether this newly identified intron 1 element is activated in concordance or discordance with the 5′ CAP proximal promoter, and whether the promoters are differentially regulated during keratinocyte growth and differentiation.

Effect of RA and Ca^{2+} on Transcriptional Activity of the TGM1 Gene

It is well established that calcium ions (Ca^{2+}) and retinoic acid (RA) are positive and negative effectors, respectively, of both keratinocyte differentiation [Fuchs and Green, 1981; Yuspa and Harris, 1974; Rice and Green, 1979] and the steady-state level of TGM1 mRNA [Floyd and Jetten, 1989]. TGM1 gene expression is regulated at the level of transcription [Yada et al., 1993; Floyd and Jetten, 1989; Michel et al., 1989], and if the TGM1 5′ end contains Ca^{2+} and RA responsive elements, CAT reporter expression directed by pK3 should be upregulated by Ca^{2+} and suppressed by RA treatment of keratinocytes. Lysates from proliferating NHK (0.1 mM Ca^{2+} medium) transfected with each construct were assayed for CAT activity and compared pair-wise to those from differentiat-

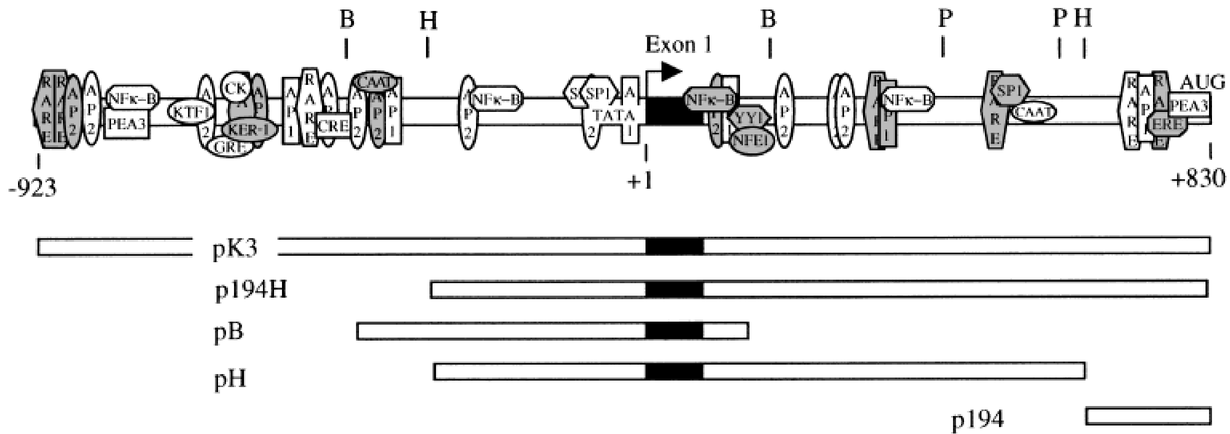

Fig. 2. Deletion mutants of the 5′ upstream regulatory region of the TGM1 gene with putative regulatory sites. Top: Schematic of the genomic DNA fragment located upstream of the translation initiation codon (AUG). This fragment (the K3 subclone) contains 923 basepairs of the untranscribed region, 94 basepairs of exon I that starts at the transcription initiation CAP site (+1) and 736 basepairs of intron 1; upper case letters represent restriction enzyme sites: X, XhoI; B, BamHI; H, HindIII; N, NlaIII; closed boxes, exon I. Shaded symbols represent overlapping consensus sequences. Bottom: Bars indicate K3 sequences present in the indicated K3-deletion mutation constructs, in the promoterless pBLCAT3 vector; closed boxes, exon I.

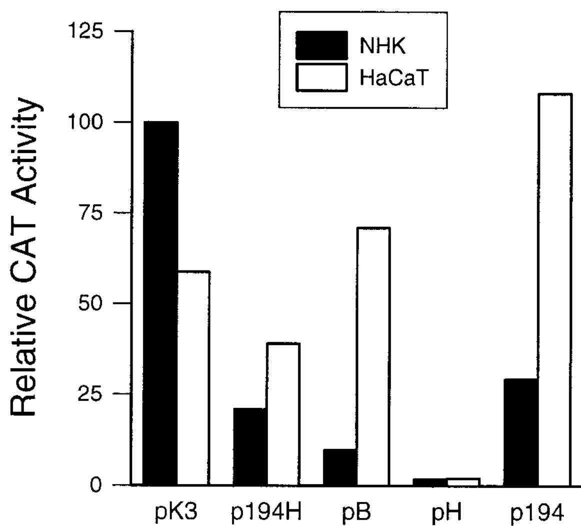

Fig. 3. CAT assay results from neonatal human keratinocytes (NHK) and HaCaT cell lysates. Cells were cultured in 0.1 mM Ca^{2+} (low Ca^{2+}) medium. Values represent percent acetylated chloramphenicol normalized to one OD_{420} unit of β-galactosidase activity, relative to the value for NHK transfected with pK3, designated as 100%. Numbers are the mean values obtained from three to seven experiments.

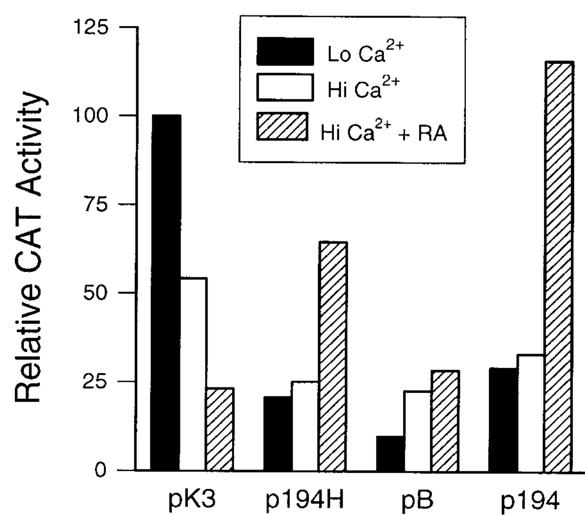

Fig. 4. Effect of retinoic acid (RA) and calcium ion (Ca^{2+}) concentrations on K3 fragment-mediated CAT reporter expression in neonatal human keratinocyte (NHK) lysates 48 h after transfection. Cells grown in low Ca^{2+} (LoCa) medium (0.1 mM) to 80% confluence were switched after transfection to medium supplemented with high (1.2 mM) Ca^{2+} (Hi Ca), or with high Ca^{2+} and 10^{-6}M *all trans* retinoic acid (RA). Values represent percent acetylated chloramphenicol normalized to one OD_{420} unit of β-galactosidase activity, relative to the value for NHK transfected with pK3, designated as 100%. Numbers are the mean values obtained from three to seven experiments. Positive (pSV2CAT) and negative (pBLCAT3) are not shown.

ing cells (1.2 mM Ca^{2+}) and to those from cells treated with 1.2 mM Ca^{2+} and 10^{-6} M RA. Contrary to expectations, high Ca^{2+} suppressed pK3-driven CAT gene expression by 50% (Fig. 4), suggesting that pK3 contains a negative Ca^{2+} responsive element(s). After deletion of intron 1 and the 5′ X/B fragment (pB plasmid), instead of supressing, high Ca^{2+} doubled the reporter activity. The p194H plasmid, lacking the 5′ X/H region, was essentially unresponsive to high Ca^{2+}. Taken together, these data suggest that a negative Ca^{2+}-response element lies 5′ of the BamHI site in the 5′ X/H region, and a positive Ca^{2+} response element between the 5′

X/H BamHI and Hind III sites. The increased CAT activity could also result from removal of a negative Ca^{2+}-responsive control element within intron 1 deleted in the pB construct. Sequence analysis of intron 1 revealed two consensus motifs, a reverse CAAT box [Connelly and Manley, 1989] and a negative RA response element (nRARE) (characterized in the rat oxytocin gene [Lipkin et al., 1992]) overlapping with an Sp1 site. Removal of these sequences in the p194HΔ construct by deletion of the internal PstI (+434 to +630) fragment enhanced CAT activity 56% relative to p194H in differentiating keratinocytes and 70% in RA-treated cells (not shown), suggesting that this region may contain a functionally significant repressor(s).

The presence of RA strongly suppressed pK3-directed CAT activity in Ca^{2+} treated cells (Fig. 4) in addition to the decrease caused by the Ca^{2+} treatment. Deletion of the 5′ X/H proximal element (p194H) reversed the RA-mediated response, from suppression to strong activation, indicating the presence of a powerful negative RA response element (RARE) in the 5′ X/H region that represses function of most of the positive transcription elements contributing to the pK3-directed CAT activity. In contrast, p194-driven CAT gene expression showed a prodigous RA mediated increase (Fig. 4), demonstrating that the intronic 194 bp fragment contains a highly effective positive RARE and can initiate efficient transcription. The TGM1 intron 1 appears therefore to occupy a regulatory function rather than acting purely as a generic enhancer of CAT gene expression.

The Proximal Region and Intron 1 of the Human TGM1 Gene Contain Two Distinct Promoters; the Proximal (P1) and Intronic (P2)

The fact that two constructs, pBH and p194, that have no overlapping TGM1 DNA sequence each express significant CAT activity in transfected NHK strongly suggests that the TGM1 gene is regulated by two promoters; the previously described proximal promoter [Mariniello et al., 1995; Yamada et al., 1994] located near the CATAA sequence, which resembles a TATA-like sequence motif, may be designated P1, and the promoter located within intron 1 as P2.

To eliminate the possibility that p194 acts to enhance a spurious promoter derived from the vector sequences, RT-PCR of RNA isolated from transiently transfected keratinocytes was performed using pairs of primers that would distinguish between specific p194 and pK3, or non-specific pUC19 derived sequences. There was no product observed following PCR based on primers #104 pUC19/#490 CAT that produce an amplified fragment only if transcription is initiated from the pUC19 sequences surrounding the insertion site. PCR using the #281TGM1/#490CAT pair of primers (pK3 transcript) or #307TGM1/#490CAT (p194 transcript) that would detect only the exogenous TGM1 spliced and unspliced transcripts produced fragments of the expected sizes, ruling out the possibility of spurious initiation of transcription (Fig. 5). In addition, all deletion mutated DNAs were subcloned into the plasmid pCATbasic, which has different sequences surrounding the insertion site. The CAT assay data were similar to those obtained with the pBLCAT3 reporter vectors (not shown), further supporting the finding that transcription can be initiated from the TGM1 intron 1 194 bp fragment as well as from the proximal promoter located upstream of exon 1.

To further distinguish the 194 bp of intron 1 as a promoter rather than an enhancer element, the 194 bp DNA fragment was subcloned into the pCATbasic vector in the reverse orientation and assayed for CAT activity. Only the forward 194 bp fragment initiated CAT gene expression (Fig. 6), indicative of promoter rather than enhancer function.

If TGM1 gene transcription is determined by two distinct promoters, and both promoters are functional, it should be possible to identify a

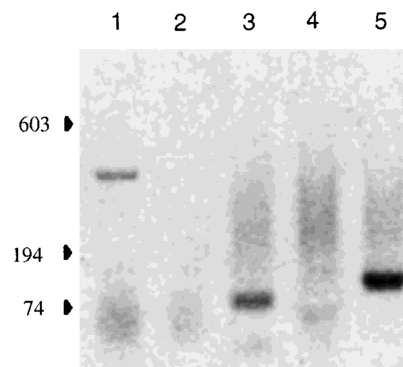

Fig. 5. RT-PCR of RNA isolated from transiently transfected keratinocytes with p194H (lanes 1,2) and with p194 (lanes 3–5). The pair of primers used were: intron 1 #547/CAT #490 (**lane 1**); pUC19 #104/ #490 (**lane 2**); intron 1 #307/ #490 (**lane 3**); intron 1 #307/ exon 2 #159 (**lane 4**); exon 1 #281/ exon 2 #159 (**lane 5**). The specificity of the assay for the spliced and unspliced transcripts was confirmed by Southern blot using the TGM1 cDNA as a probe (not shown).

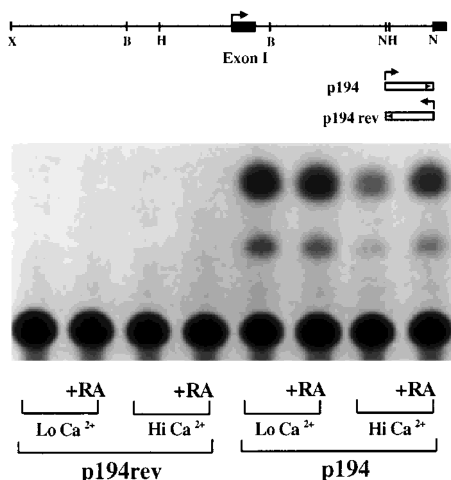

Fig. 6. Orientation dependence of the 194 bp K3 fragment, in the p194 construct, on CAT reporter trancription in NHK. Arrows on the open bar schematics indicate the orientation of the 194 element. The autoradiograph shows CAT assays of transiently transfected NHK cultured in low (0.1 mM) Ca^{2+} and high (1.2 mM) Ca^{2+} in the presence and absence of 10^{-6}M RA.

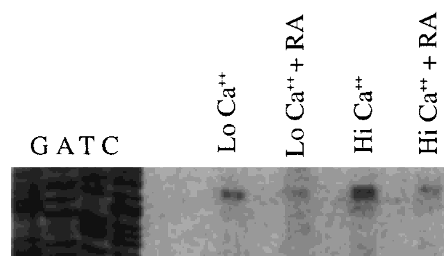

Fig. 7. RNase protection assay performed with intron 1 RNA probe. Total RNA from neonatal human keratinocytes, grown in low (0.1 mM) Ca^{2+} in the absence (NHK) or presence (NHK+RA) of 10^{-6} M RA, was hybridized to in vitro transcribed 230 bp probe containig 194 nucleotides of intron 1. Sequencing reactions from an irrelevant template were run in parallel as a size standard. GATC, order of the sequencing reaction termination dideoxynucleotides. Other controls performed but not shown include running undigested probe, RNase digestion of unhybridized RNA, and RNase digestion of RNA hybridized to actin RNA probe.

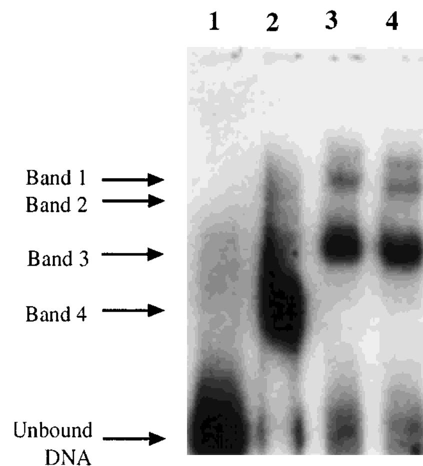

Fig. 8. Gel retardation assay. Nuclear extracts were isolated from NHK cultured in low Ca^{2+} (0.1 mM) (**lane 1**), low Ca^{2+} (0.1 mM) plus 10^{-6}M RA (**lane2**), high (1.2 mM) Ca^{2+} plus 10^{-6}M RA (**lane 3**), and high (1.2 mM) Ca^{2+} (**lane 4**).

transcript containing intron 1-specific sequences. To identify this transcript, an RNase protection assay was performed with in vitro transcribed RNA p194 probe. The labeled probe was hybridized to total RNA isolated from keratinocytes grown under different conditions. The intron 1 specific 230 nucleotide long probe protected a 170 bp fragment from RNase digestion (Fig. 7), positively identifying intron 1-containing RNA and supporting the conclusion that two distinct transcripts are expressed in keratinocytes from the human TGM1 gene. It is still possible that the two transcripts result from alternative splicing rather than from initiation by two promoters, a point that will ultimately be resolved by mapping of the transcription start sites by 5′ RACE for example.

Keratinocytes Contain Nuclear Proteins That Interact With the Intron 1 Promoter Region of the Human TGM1 Gene

To determine whether the 194 bp DNA fragment has functional significance in vivo, this TGM1 intron 1 fragment was analyzed for DNA-protein binding activity using nuclear extracts from keratinocytes grown under differentiating and proliferating conditions in the presence and absence of RA. The electrophoretic mobility shift gel presented in Figure 8 shows that the 194 pb TGM1 fragment interacts with specific *trans*-acting factors present in some nuclear extracts but not in others. Band 4 was formed only with extracts of proliferating keratinocytes while bands 1, 2, and 3 were detected only with nuclear extracts of differentiating cells. Interestingly, nuclear extracts of keratinocytes cultured in high Ca^{2+} medium supplemented with 10^{-6}M RA do not form the DNA-protein complex designated as band 1, suggesting that RA inhibits either interaction or synthesis of a putative regulatory protein. Although more detailed studies are warranted, these data support the assumption that the 194 bp DNA fragment of the first intron contains *cis*-acting regulatory sequences that bind proteins specific to the particular physiologic state of keratinocytes.

DISCUSSION

Transglutaminase type 1 activity increases with the onset of keratinocyte differentiation [Thacher and Rice, 1985] and is Ca^{2+}-inducible in culture as is differentiation generally, and therefore the TGM 1 gene product is often cited as a marker of this process. The temporal and spatial patterns of RNA and the protein cellular and subcellular distribution vary during development [Polakowska et al., 1994; Nonomura et al., 1993], between keratinocyte growth conditions [Saunders et al., 1993; Steinert et al., 1996; Yada et al., 1993], and between keratinizing epithelia of different sites of origin and of different species [Schroeder et al., 1992; Michel et al., 1992], indicating that regulatory mechanisms involved in expression of the active TGase 1 are complex and may operate at all levels of genetic control.

To determine the molecular mechanisms controlling transcriptional activity of the TGM1 gene during keratinocyte differentiation that can be mimicked [Green, 1979; Fuchs, 1990] and modulated in vitro in response to Ca^{2+} and RA concentrations in the growth medium [Blumenberg and Tomic-Canic, 1997; Fuchs, 1990], functional mapping of the TGM1 5′ end region, using a CAT reporter gene assay, was performed in human keratinocytes and fibroblasts. Several findings underline the importance of intron 1 in regulating transcriptional activity of the human TGM1 gene as a component of a transcription repression mechanism. It was found that the 1753 bp upstream of the ATG translation initiation codon of this gene (pK3 construct) directed high CAT activity in undifferentiated keratinocytes. The maximal activity depended on the presence of both the 5′X/H element in the proximal 5′ upstream regulatory region (5′URR) and the first intron. Neither the 5′X/H in p3.0(-)int (Fig. 1) nor the intron 1 in p194H (Fig. 3) was as effective in directing CAT activity as when acting in union in the p3.0(+)int and pK3 constructs, demonstrating the functional interdependence of the URR and intron 1 *cis*-regulatory elements and their cooperative contribution to the overall transcriptional activity of the human TGM1 gene. This 5′URR and intron 1 interdependent enhancing activity in undifferentiated NHK could be, however, suppressed by high Ca^{2+} and RA in NHK (Fig. 4) and was downregulated in HaCaT cells (Fig. 3). The inhibitory effect appeared to be mediated again by interdependent 5′X/H and intron 1 *cis*-acting elements.

The 5′X/H transcription control element was strongly repressed by RA. The RA-induced silencing of the 5′X/H element was dominant over the RA stimulation of intron 1 activity seen in the absence of the 5′X/H element in p194H (Fig. 4). Inspection of the 5′X/H sequence reveals a direct repeat 5 (DR5) type of RARE [for reviews see Chambon, 1996; Fisher and Voorhees, 1996] mediating retinoic acid receptor (RAR)-signaling. In keratinocytes, negative regulation of RARE activity is attributed to signaling through RARγ [Miquel et al., 1992; Aneskievich and Fuchs, 1992], the most abundant RAR in skin [Fisher and Voorhees, 1996]. It is therefore likely that the RA-induced repression of the TGM1 gene is exerted by binding of this receptor to the putative DR5 RARE within the 5′X/H transcription control element. The recent finding that RARγ was instrumental in suppression of a differentiation-specific keratin gene promoter [Aneskievich and Fuchs, 1995] supports this assumption. Another mechanism by which RA can repress 5′X/H activity may be through antagonizing the activity of its two AP-1 sites [for review see Fisher and Voorhees, 1996] or through certain functional AP-2 sites [Mariniello et al., 1995]. In keeping with the latter possibility, AP-2 gene expression in keratinocytes was found to be downregulated by RA [Wanner et al., 1996]. In addition to RA, the 5′X/H element apparently contains regulatory sequences that control the TGM1 gene transcription in response to high Ca^{2+}. This element was repressed in Ca^{2+}-induced differentiating NHK (Fig. 4) and was inactive in the cellular context of HaCaT cells (Fig. 3), which require high Ca^{2+} for growth but fail to exhibit Ca^{2+}-induced differentiation. This cell type-specific Ca^{2+}-responsiveness appears to be controlled by two subelements of the 5′X/H region. A silencer located upstream of the BamHI site was required for the strong Ca^{2+} suppression of pK3 activity, and an enhancer of Ca^{2+}-signaling located downstream of the BamHI site was detected by the increased pB-directed CAT activity, particularly evident in the HaCaT cell background. Both subelements contain a large number of diverse regulatory motifs (Fig. 2) that have the potential to bind transcription factors implicated in controlling expression of keratinocyte differentiation-spe-

cific genes [reviewed in Eckert et al., 1997a]. Interestingly, the only Ca^{2+}-enhanced transcriptional activity of the TGM1 gene observed in our study coincided with the absence of most of intron 1 in the pB plasmid, suggesting that the deleted sequences may constitute an intronic negative regulatory element that silenced the 5′URR enhancer subelement, once again demonstrating the functional interdependence of 5′URR and intronic regulatory elements of the human TGM1 gene. In addition, the intron 1 region could act, depending on the cell type, as a silencer (HaCaT) or enhancer (undifferentiated NHK) of CAT gene expression (Fig. 3). In this study, two distinct regions were identified within intron 1 that could be involved in the repression or induction of TGM1 gene expression; the PstI/PstI negative element which contains a reverse CCAAT box and a short nRARE element [Lipkin et al., 1992] overlapping an Sp1 site, which may have a role in repression of transcription [Chung et al., 1995], and the 194 bp element, which displayed promoter activity.

Several properties that are characteristic of a promoter element, including initiation of CAT gene expression in an orientation-dependent fashion (Fig. 6), RA-signaled inducibility (Fig. 4), presence of a distinct transcript (Fig. 7), and the DNA-nuclear protein binding activity (Fig. 8), support the notion that the 194 bp region of intron 1 may function under unidentified conditions as a P2 promoter, alternative to the P1 core promoter in the 5′URR [Mariniello et al., 1995; Yamanishi et al., 1992] of the TGM1 gene. The intronic P2 promoter has the potential to be functionally active, since intron 1 contains many putative regulatory flanking elements (Fig. 2), some apparently functional (Fig. 8), and some, like Sp1, AP-1, and AP-2, common to the 5′ URR. So in addition to the environmentally modulated interplay of its regulatory transcription elements with 5′URR elements, the intron 1 region may contribute to differential TGM1 gene expression as an alternative promoter.

A regulatory role of the first intron has been demonstrated for many genes [Kozak, 1991], including keratinocyte-expressed involucrin [Carroll and Taichman, 1992] and keratin 18 [Umezawa et al., 1997], genes which are regulated in a tissue-, developmental-, or differentiation-specific manner in response to environmental cues. Examples of the interdependence between intron 1 and 5′URR elements in gene regulation are documented [Hadden et al., 1997; Storbeck et al., 1998; Tiffany et al., 1996], and intron 1 functional significance was confirmed for the type I collagen gene in transgenic mice [Hormuzdi et al., 1998]. Similarly, utilization of alternative promoters is a widespread phenomenon in eukaryotes, and like intron 1 regulatory elements, alternative promoters are instrumental in generating stage-, temporal-, and tissue-specific diversity of gene expression in response to different signaling pathways [Ayoubi and Van De Ven, 1996; Kozak, 1991]. If human TGM1 gene expression is controlled by alternative promoters, usage of the intronic promoter would result in omission of the untranslated exon I, and in the production of transcripts with alternative leader exons, which could affect stability and translatability, as well as subcellular distribution of the message and TGase 1 [Ayoubi and Van De Ven, 1996]. Also, the utilization of multiple promoters provides an additional level of gene expression control, by transcriptional interference of alternative promoters, or occlusion of downstream promoters, as seen in several prokaryotic and eukaryotic genes [Irniger et al., 1992], including PCNA [Alder et al., 1992], c-fos [Mechti et al., 1991], and human Fatty-acid Synthase [Hsu et al., 1996]. The reverse CCAAT box found in the Pst1 fragment of intron 1 may function, like in other genes [Eggermont and Proudfoot, 1993; Connelly and Manley, 1989], as a negative element in overall transcriptional activity of the human TGM1 gene, by directing transcriptional arrest to prevent interference of the P1 with the P2 promoter. Deletion of this CCAAT box in p194HΔ construct increased CAT activity suggestive of such negative regulatory role, but its functional significance has yet to be established.

On the basis of the data presented, we propose that in undifferentiated keratinocyte transcriptional activity of the human TGM1 gene is controlled by a default mechanism in the absence of regulatory signals. This mechanism can be repressed by cell-, growth-, or stratum-specific regulatory signals that control TGM1 differential expression. These signals would include, integrated into a default mechanism, the interplay of the 5′X/H and intron 1 transcription control elements. These elements, in response to intrinsic and extrinsic factors including Ca^{2+} and RA, can be activated in expressing or inactivated in non-expressing cells. As shown

by us and other investigators, TGM1 gene expression can be regulated also by TPA [Saunders et al., 1993; Liew and Yamanishi, 1992; Yada et al., 1993], by signaling pathways involving protein kinase Cη (PKCη) [Ueda et al., 1996], retinoid receptor RXR/RAR heterodimer [Yamada et al., 1994], the AP-2 [Mariniello et al., 1995], and by the AP-1 c-jun and c-fos complex [Yamada et al., 1994; Rossi et al., 1998]. The 5′ proximal and intronic regions of the human TGM1 gene share multiple keratinocyte- and differentiation-specific *cis*-acting elements (Fig. 2) including functionally active AP-2 [Mariniello et al., 1995], Sp1 [Doi et al., 1996], and AP-1 [Rossi et al., 1998] binding sites, which together may contribute by the interplay to the spatial-, temporal-, and tissue-specific but also species-specific expression of the TGM1 gene. Their functional interdependence is apparently not instrumental in the regulation of the rabbit TGM1 gene expression [Saunders et al., 1993], indicating that the human TGM1 gene appears to be regulated in a different and species-specific manner. Many observations support this concept. Rabbit TGM1 cDNA, although highly homologous to human, has very little homology in intron 1 sequences, lacking preservation of the consensus transcription factor binding motifs. Also, the human TGM1 5′ proximal promoter was inactive in human primary, SCC-9, or SIK keratinocytes (Fig. 1 and [Mariniello et al., 1995]) while highly active in rat cells [Mariniello et al., 1995; Ueda et al., 1996] in which the regulatory function of intron 1 may not occur. We further found that the TGM1 5′untranslated region efficiently drives β-gal transgene expression in human keratinocytes in vitro but was inactive in trangenic mice (data not shown), whereas the TGM1 transgene devoid of intron 1 was successfully expressed in mice [Yamada et al., 1997], suggesting again a species-specificity of the regulatory machinery, which occurs in other genes as well [Gaunitz et al., 1997; Oskouian et al., 1997]. It may be said that the role of the intron 1 sequences is to tune the human TGM1 gene expression to the particular cell type or state of a cell.

The physiological role of the TGM1 interdependent transcription control elements of the 5′ URR and intron 1, including utilization of alternative promoters, is not yet known. In vivo, TGase 1 activity is limited to the narrow band of terminally differentiating epidermal cells in the stratum granulosum [Michel and Demarchez, 1988], while the TGM1 gene is transcriptionally and translationally active earlier, in suprabasal [Nonomura et al., 1993; Michel et al., 1992] or even in proliferating basal [Schroeder et al., 1992; Parenteau et al., 1986] cell layers. Both keratinocyte differentiation and TGM1 gene expression appear to be controlled by the affect of opposing Ca^{2+} and retinoic acid (RA) concentration gradients, existing in the native epidermis [Menon and Elias, 1991; Fisher and Voorhees, 1996; Vicanova et al., 1998], on intracellular levels. Low Ca^{2+} and high RA concentrations in the stratum basale permit basal cell proliferation. In vitro low Ca^{2+} conditions permit operation of a default mechanism in basal-like undiferentiated keratinocytes. The TGM1 P1 promoter region containing CpG islands created by two Sp1 and eight AP-2 recognition sites implicated in regulation of several keratinocyte-specific genes [Wu et al., 1994; Mariniello et al., 1995; Lee et al., 1996; Eckert et al., 1997], displays characteristics of a housekeeping gene which has the genetically determined potential to be active in undifferentiated cells [Tyndall et al., 1992]. Thus a default mechanism most likely would direct TGM1 gene expression in the basal cell layer if it were not silenced by, at least, a high level of RA in this layer. As demonstrated above, instrumental in suppression by RA appears to be the 5′X/H element. Topical RA treatment in vivo had no effect on the TGM1 mRNA steady state level [Griffiths et al., 1992], reflecting perhaps that the gene is already repressed in basal cells. According to our model the precocious TGM1 mRNA expression observed in psoriasis [Schroeder et al., 1992; Bernard et al., 1986], or in basal keratinocytes of other keratinizing tissues [Yamada et al., 1997; Parenteau et al., 1986; Schroeder et al., 1992], results from derepressing conditions, which may involve abnormally functioning RA-signaling or metabolism. This would explain the effectiveness of RA in treatment of psoriatic plaques [Orfanos et al., 1997; McClelland, 1997] and normalization of TGM1 gene expression [Nagpal et al., 1996]. In normal epidermis the decreasing level of RA accompanied by the gradually increasing Ca^{2+} concentration stimulates keratinocyte differentiation in suprabasal cell layers and TGM1 mRNA accumulation [Fuchs, 1990; reviewed in Saunders et al., 1993; Eckert et al., 1997]. This accumulation was downregulated by RA in a dose dependent manner [Saunders et al., 1993],

demonstrating that transcription regulatory elements of the TGM1 may be sensing the in vivo RA concentration. Responsiveness of TGM1 regulatory elements to increasing Ca^{2+} concentration in vivo and in vitro is less direct. In vitro, steady state TGM1 mRNA increases to a maximum 3.6-fold at 24h in human keratinocytes [Gibson et al., 1996] and to 17-fold in 24 h in mouse keratinocytes [Dlugosz and Yuspa, 1994] after Ca^{2+} induction of differentiation. Subsequently it falls to the un-induced level, while the processes of differentiation, including TGase 1-catalyzed cell envelope formation [Pillai et al., 1990; reviewed in Reichert et al., 1993], continues to advance. It appears then, that the TGM1 gene is transcriptionally active at early stages of keratinocyte differentiation, a conclusion also reached by [Su et al., 1994]. Later, in cells of more superficial layers, when the Ca^{2+} concentration is high, transcriptional activity of the TGM1 gene in differentiating keratinocytes is perhaps downregulated by silencing or dispatching the interdependent positive function of the 5′URR and intron 1 transcriptional control elements. High Ca^{2+} may be necessary for the posttranscriptional regulation of TGM1 gene expression evident by the discordant patterns of mRNA and protein distribution observed in vivo [Thacher, 1989; Griffiths et al., 1992] and in vitro [Gibson et al., 1996] and thought to occur in these layers [Gibson et al., 1996]. Thus the reduced CAT activity under high Ca^{2+} conditions, directed by the pK3 construct, is not surprising and reflects decreased TGM1 transcriptional activity in differentiated cells of layers with high Ca^{2+}. Inactivation of the TGM1 transcriptional activity in terminally differentiating cells with less accessible chromatin structure is logical, and perhaps its regulation at the posttranscriptional level may provide the advantage of a quick response to extracellular or intracellular signaling.

In summary it appears that human TGM1 gene expression in human epidermal keratinocytes is regulated by a default mechanism integrating the cooperatively operating 5′URR and intronic alternative promoters, controlled by the cell- and growth-specific interplay of their regulatory elements. In vivo the transcriptional activity of the TGM1 gene is downregulated in basal cells, perhaps due to the high RA concentration, and is upregulated upon leaving the basal cell layer. This suggests that the TGM1 gene is activated in a spatially and temporally narrow window before Ca^{2+} reaches high levels and after RA falls to a concentration permitting its expression. RA appears to be a dominant repressing factor of the TGM1 default mechanism of transcription.

ACKNOWLEDGMENTS

We thank Dr. Bo Lu for construction of the p3.0(-)int and p3.0(+)int plasmids.

REFERENCES

Aeschlimann D, Koeller MK, Allen-Hoffmann BL, Mosher DF. 1998. Isolation of a cDNA encoding a novel member of the transglutaminase gene family from human keratinocytes. J Biol Chem 273:3452–3460.

Aeschlimann D, Paulsson M. 1994. Transglutaminases: Protein cross-linking enzymes in tissues and body fluids. Thrombosis Haemostasis 71:402–415.

Alder H, Yoshinouchi M, Prystowsky MB, Appasamy P, Baserga R. 1992. A conserved region in intron 1 negatively regulates the expression of the PCNA gene. Nucleic Acids Res 20:1769–1775.

Aneskievich BJ, Fuchs E. 1992. Terminal differentiation in keratinocytes involves positive as well as negative regulation by retinoic acid receptors and retinoid X receptors at retinoid response elements. Mol Cell Biol 12:4862–4871.

Aneskievich BJ, Fuchs E. 1995. The A/B domain of truncated retinoic acid receptors can block differentiation and promote features of malignancy. J Cell Science 108:195–205.

Ayoubi TA, Van De Ven WJ. 1996. Regulation of gene expression by alternative promoters. FASEB J 10:453–460.

Bernard BA, Reano A, Darmon YM, Thivolet J. 1986. Precocious appearance of involucrin and epidermal transglutaminase during differentiation of psoriatic skin. Br J Dermatol 114:279–283.

Blumenberg M, Tomic-Canic M. 1997. Human epidermal keratinocyte: Keratinization processes. EXS 78:1–29.

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106:761–771.

Carroll JM, Taichman L. 1992. Characterization of the human involucrin promoter using a transient β-galactosidase assay. J Cell Sci 103:925–930.

Chambon P. 1996. A decade of molecular biology of retinoic acid receptors. FASEB J 10:940–954.

Choate KA, Khavari PA. 1997. Direct cutaneous gene delivery in a human genetic skin disease. Hum Gene Ther 8:1659–1665.

Chung DC, Brand SJ, Tillotson LG. 1995. Mutually exclusive interactions between factors binding to adjacent Sp1 and AT-rich elements regulate gastrin gene transcription in insulinoma cells. J Biol Chem 270:8829–8836.

Connelly S, Manley JL. 1989. A CCAAT box sequence in the adenovirus major late promoter functions as part of an RNA polymerase II termination signal. Cell 57:561–571.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiated by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1479.

Dlugosz AA, Yuspa SH. 1994. Ptotein kinase C regulates keratinocyte transglutaminase (TGK) gene expression in cultured primary mouse epidermal keratinocytes induced to terminally differentiate by calcium. J Invest Dermatol 102:409–414.

Doi H, Ueda E, Yamanishi K, Fujii Y, Kamoda H, Yasuno H. 1996. Transcriptional regulation of human transglutaminase 1 gene by Sp1. J Clin Exp Med 177:281–282.

Eckert RL, Crish JF, Banks EB, Welter JF. 1997a. The epidermis: Genes on – genes off. J Invest Dermatol 109: 501–509.

Eckert RL, Crish JF, Robinson NA. 1997b. The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. Physiol Rev 77:397–424.

Eggermont J, Proudfoot NJ. 1993. Poly(A) signals and transcriptional pause sites combine to prevent interference between RNA polymerase II promoters. EMBO J 12:2539–2548.

Fesus L, Davies PJA, Piacentini M. 1991. Apoptosis: Molecular mechanisms in programmed cell death. Eur J Cell Biol 56:170–177.

Fisher GJ, Voorhees JJ. 1996. Molecular mechanisms of retinoid actions in skin. FASEB J 10:1002–1013.

Floyd EE, Jetten AM. 1989. Regulation of type I (epidermal) transglutaminase mRNA levels during squamous differentiation: Down regulation by retinoids. Mol Cell Biol 9:4846–4851.

Folk JE. 1996. Transglutaminases. Annu Rev Biochem 49:517–531.

Fuchs E. 1990. Epidermal differentiation: The bare essentials. J Cell Biol 111:2807–2814.

Fuchs E, Green H. 1981. Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. Cell 25:617–625.

Gaunitz F, Gaunitz C, Papke M, Gebhardt R. 1997. Cis regulatory sequences from the first intron of the rat glutamine synthetase gene are involved in hepatocyte specific expression of the enzyme. Biol Chem 378:11–18.

Gibson DFC, Ratnam AV, Bikle DD. 1996. Evidence for separate control mechanisms at the message, protein, and enzyme activation levels for transglutaminase during calcium-induced differentiation of normal and transformed human keratinocytes. J Invest Dermatol 106:154–161.

Green H. 1979. The keratinocyte as differentiated cell type. Harvey Lectures 74:101–139.

Greenberg CS, Birckbichler PJ, Rice RH. 1991. Transglutaminases: Multifunctional cross-linking enzymes that stabilize tissues. FASEB J 5:3071–3077.

Griffiths CEM, Rosenthal DS, Reddy AP, Elder JT, Astrom A, Leach K, Wang TS, Finkel LJ, Yuspa SH, Voorhees JJ, Fisher GJ. 1992. Short-term retinoic acid treatment increases in vivo, but decreases in vitro, epidermal transglutaminase-K enzyme activity and immunoreactivity. J Invest Dermatol 99:283–288.

Haake AR, Polakowska RR. 1993. Cell death by apoptosis in epidermal biology. J Invest Dermatol 101:107–112.

Hadden TJ, Ryou C, Miller RE. 1997. Elements in the distal 5′-flanking sequence and the first intron function cooperatively to regulate glutamine synthetase transcription during adipocyte differentiation. Nucleic Acids Res 25: 3930–3936.

Hennies HC, Kuster W, Weibe V, Krebsova A, Reis A. 1998. Genotype/Phenotype correlation in autosomal recessive lamellar ichthyosis. Am J Human Genetics 62:1052–1061.

Hohl D. 1990. Cornified cell envelope. Dermatologica 180: 201–211.

Hormuzdi SG, Penttinen R, Jaenisch R, Bornstein P. 1998. A gene-targeting approach identifies a function for the first intron in expression of the ·1(I) collagen gene. Mol Cell Biol 18:3368–3375.

Hsu MH, Chirala SS, Wakil SJ. 1996. Human fatty acid synthase gene. Evidence for the presence of two promoters and their functional interaction. J Biol Chem 271: 13584–13592.

Huber M, Rettler I, Bernasconi K, Frenk E, Lavrijsen SPM, Ponec M, Bon A, Lautenschlager S, Schorderet DF, Hohl D. 1995. Mutations of keratinocyte transglutaminase in Lamellar Ichthyosis. Science 267:525–528.

Irniger Egli CM, Kuenzler M, Braus GH. 1992. The yeast actin intron contains a cryptic promoter that can be switched on by preventing transcriptional interference. Nucleic Acids Res 20:4733–4739.

Kim I-G, McBride OW, Wang M, Kim S-Y, Idler WW, Steinert PM. 1992. Structure and organization of the human transglutaminase 1 gene. J Biol Chem 267:7710–7717.

Kim I-G, Lee S-C, Lee J-H, Yang J-M, Chung S-I, Steinert PM. 1994. Structure and organization of the human transglutaminase 3 gene: Evolutionary relationship to the transglutaminase family. J Invest Dermatol 103:137–142.

Kozak M. 1991. An analysis of vertebrate mRNA sequences: Intimations of translational control. J Cell Biol 115:887–903.

Lee J-H, Jang S-I, Yang J-M, Markova NG, Steinert PM. 1996. The proximal promoter of the human transglutaminase 3 gene. J Biol Chem 271:4561–4568.

Liew F-M, Yamanishi K. 1992. Regulation of transglutaminase 1 gene expression by 12-O-tetradecanoylphorbol-13-acetate, dexamethasone, and retinoic acid in cultured human keratinocytes. Exp Cell Res 202:310–315.

Lipkin SM, Nelson CA, Glass CK, Rosenfeld MG. 1992. A negative retinoic acid response element in the rat oxytocin promoter restricts transcriptional stimulation by heterologous transactivation domains. Proc Natl Acad Sci USA 89:1209–1213.

Lorand L, Conrad SM. 1984. Transglutaminases. Mol Cell Biochem 58:9–35.

Luckow B, Schutz G. 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids Res 15:5490.

Mariniello L, Qin Q, Jessen BA, Rice RH. 1995. Keratinocyte transglutaminase promoter analysis. J Biol Chem 270:31358–31363.

Matsuki M, Yamashita F, Ishida-Yamamoto A, Yamada K, Kinoshita C, Fushiki S, Ueda E, Morishima Y, Tabata K, Yasuno H, Hashida M, Iisuka H, Ikawa M, Okabe M, Kondoh G, Kinoshita T, Takeda J, Yamanishi K. 1998. Defective stratum corneum and early neonatal death in mice lacking the gene for transglutaminase 1 (keratinocyte transglutaminase). Proc Natl Acad Sci USA 95:1044–1049.

McClelland PB. 1997. New treatment options for psoriasis. Dermatol Nurs 9:295–304.
Mechti N, Piechaczyk M, Blanchard J-M, Jeanteur P, Lebleu B. 1991. Sequence requirements for premature transcription arrest within the first intron of the mouse c-fos gene. Mol Cell Biol 11:2832–2841.
Menon GK, Elias PM. 1991. Ultrastructural localization of calcium in psoriatic and normal human epidermis. Arch Dermatol 127:57–63.
Michel S, Reichert S, Isnard U, Shroot B, Schmidt R. 1989. Retinoic acid controls expression of epidermal transglutaminase at the pre-translational level. FEBS Lett 258: 35–38.
Michel S, Bernerd F, Jetten AM, Floyd EE, Shroot B, Reichert U. 1992. Expression of keratinocyte transglutaminase mRNA revealed by in situ hybridization. J Invest Dermatol 98:364–368.
Michel S, Demarchez M. 1988. Localization and in vivo activity of epidermal transglutaminase. J Invest Dermatol 90:472–474.
Miquel C, Clusel C, Semat A, Gerst C, Darmon M. 1992. Retinoic acid receptor isoform RARg1: An antagonist of the transactivation of the RAR·RARE in epithelial cell lines and normal human keratinocytes. Mol Biol Rep 17:35–45.
Nagpal S, Thacher SM, Patel S, Friant S, Malhotra M, Shafer J, Krasinski G, Asano AT, Teng M, Duvic M, Chandraratna RA. 1996. Negative regulation of two hyperproliferative keratinocyte differentiation markers by a retinoic acid receptor-specific retinoid: Insight into the mechanism of retinoid action in psoriasis. Cell Growth Differ 7:1783–1791.
Nakaoka H, Perez DM, Baek KJ, Das T, Husain A, Misono K, Im M-J, Graham RM. 1994. G_h: A GTP-binding protein with transglutaminase activity and receptor signaling function. Science 264:1593–1596.
Nonomura K, Yamanishi K, Hosokawa Y, Doi H, Hirano J, Fukushima S, Yasuno H. 1993. Localization of transglutaminase 1 mRNA in normal and psoriatic epidermis by non-radioactive in situ hybridization. Br J Dermatol 128: 23–28.
Orfanos CE, Zouboulis CC, Almond-Roesler B, Geilen CC. 1997. Current use and future potential role of retinoids in dermatology. Drugs 53:358–388.
Oskouian B, Rangan B, Smith S. 1997. Regulatory elements in the first intron of the rat fatty acid synthase gene. Biochem J 324:113–121.
Parenteau NL, Pilato A, Rice RH. 1986. Induction of keratinocyte type-I transglutaminase in epithelial cells of the rat. Differentiation 33:130–141.
Phillips MA, Stewart BE, Rice RH. 1992. Genomic structure of keratinocyte transglutaminase. J Biol Chem 267: 2282–2286.
Pillai S, Bikle DD, Mancianti M-L, Cline P, Hincenbergs M. 1990. Calcium regulation of growth and differentiation of normal human keratinocytes: Modulation of differentiation competence by stages of growth and extracellular calcium. J Cell Physiol 143:294–302.
Polakowska RR, Eddy RL, Shows TB, Goldsmith LA. 1991. Epidermal type I transglutaminase (TGM1) is assigned to human chromosome 14. Cytogenet Cell Genet 56:105–107.
Polakowska RR, Eikbush T, Falciano V, Razvi F, Goldsmith LA. 1992. Organization and evolution of the human epidermal keratinocyte transglutaminase I gene. Proc Natl Acad Sci USA 89:4476–4480.
Polakowska RR, Piacentini M, Bartlett R, Goldsmith LA, Haake AR. 1994. Apoptosis in human skin development: Morphogenesis, periderm, and stem cells. Dev Dyn 199: 176–188.
Polakowska RR, Goldsmith LA. 1991. The cell envelope and transglutaminases. In: Goldsmith LA, editor. Physiology, biochemistry, and molecular biology of the skin. New York: Oxford University Press. p 168–201.
Reichert U, Michel S, Schmidt R. 1993. Molecular biology of the skin. In: Darmon M, Blumenberg M, editors. London: Academic Press, Inc. p 107–150.
Rice RH, Mehrpouyan M, O'Callahan W, Parenteau NL, Rubin GM. 1992. Keratinocyte transglutaminase: Differentiation marker and member of an extended family. Epithel Cell Biol 1:128–137.
Rice RH, Green H. 1979. Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: Activation of the cross-linking by calcium ions. Cell 18:681–694.
Roop D. 1995. Defects in the barier. Science 267:474–475.
Rose JK, Buonocore L, Whitt MA. 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. Biotechniques 10:520–525.
Rossi A, Jang S-I, Ceci R, Steinert PM, Markova NG. 1998. Effect of AP1 transcription factors on the regulation of transcription in normal human epidermal keratinocytes. J Invest Dermatol 110:34–40.
Saunders NA, Bernacki SH, Vollberg TM, Jetten AM. 1993. Regulation of transglutaminase type 1 expression in squamous differentiating rabbit tracheal epithelial cells and human epidermal keratinocytes: Effects of retinoic acid and phorbol esters. Mol Endocrinol 7:387–398.
Schroeder WT, Thacher SM, Stewart-Galetka S, Annarella M, Chema D, Siciliano MJ, Davies PJA, Tang H-Y, Sowa BA, Duvic M. 1992. Type I keratinocyte transglutaminase: Expression in human skin and psoriasis. J Invest Dermatol 99:27–34.
Steinert PM, Chung S-I, Kim S-Y. 1996. Inactive zymogen and highly active proteolytically processed membrane-bound forms of the transglutaminase 1 enzyme in human epidermal keratinocytes. Biochem Biophys Res Commun 221:101–106.
Storbeck CJ, Sabourin LA, Waring JD, Korneluk RG. 1998. Definition of regulatory sequence elements in the promoter region and the first intron of the myonic dystrophy protein kinase gene. J Biol Chem 273:9139–9147.
Su MJ, Bikle DD, Mancianti ML, Pillai S. 1994. 1,25-dihydroxyvitamin D3 potentiates the keratinocyte response to calcium. J Biol Chem 269:14723–14729.
Thacher SM. 1989. Purification of keratinocyte transglutaminase and its expression during squamous differentiation. J Invest Dermatol 89:578–584.
Thacher SM, Rice RH. 1985. Keratinocyte-specific transglutaminase of cultured human epidermal cells: Relation to cross-linked envelope formation and terminal differentiation. Cell 40:677–683.
Tiffany HL, Handen JS, Rosenberg HF. 1996. Enhanced expression of the eosinophil-derived neurotoxin ribonuclease (RNS2) gene requires interaction between the promoter and intron. J Biol Chem 271:12387–12393.

Tyndall C, Watt FM, Molloy PL, Vincent PC, Frommer M. 1992. Binding of proteins from embryonic and differentiated cells to a bidirectional promoter contained within a CpG island. J Mol Biol 226:289–299.

Ueda E, Ohno S, Kuroki T, Livneh E, Yamada K, Yamanishi K, Yasuno H. 1996. The n isoform of protein kinase C mediates transcriptional activation of the human transglutaminase 1 gene. J Biol Chem 271:9790–9794.

Umezawa A, Yamamoto H, Rhodes K, Klemsz MJ, Maki RA, Oshima RG. 1997. Methylation of an ETS site in the intron enhancer of the keratin 18 gene participates in tissue-specific repression. Mol Cell Biol 17:4885–4894.

Vicanova J, Boelsma E, Mommass AM, Kempenaar JA, Forslind B, Pallon J, Egelrud T, Koerten HK, Ponec M. 1998. Normalization of epidermal calcium distribution profile in reconstructed human epidermis is related to improvement of terminal differentiation and stratum corneum barrier formation. J Invest Dermatol 111:97–106.

Vollberg TM, Nervi C, George MD, Fujimoto W, Krust A, Jetten AM. 1992. Retinoic acid receptors as regulators of human epidermal keratinocyte differentiation. Mol Endocrinol 6:667–676.

Wanner R, Zhang J, Henz BM, Rosenbach T. 1996. AP-2 gene expression and modulation by retinoic acid during keratinocyte differentiation. Biochem Biophys Res Commun 223:666–669.

Wu RL, Chen TT, Sun TT. 1994. Functional importance of an Sp1- and an NFkappaB-related nuclear protein in a keratinocyte-specific promoter of rabbit K3 keratin gene. J Biol Chem 269:28450–28459.

Yada Y, Polakowska RR, Okano Y, Nozawa Y. 1993. Protein kinase C-dependent expression of type I transglutaminase mRNA in ganglioside GQ1b- and calcium-stimulated human keratinocytes. Biochem Biophys Res Commun 190:688–694.

Yamada K, Yamanishi K, Kakizuka A, Kibe Y, Doi H, Yasuno H. 1994. Transcriptional regulation of human transglutaminase1 gene by signaling systems of protein kinase C, RAR/RXR and Jun/Fos in keratinocytes. Biochem Mol Biol Int 34:827–836.

Yamada K, Matsuki M, Morishima Y, Ueda E, Tabata K, Yasuno H, Suzuki M, Yamanishi K. 1997. Activation of the human transglutaminase 1 promoter in transgenic mice: Terminal differentiation specific expression of the TGM1lacZ transgene in keratinized stratified squamous epithelia. Human Mol Gen 6:2223–2231.

Yamanishi K, Inazawa J, Liew F, Nonomura K, Ariyama T, Yasuno H, Abe T, Doi J, Hirano J, Fukushima S. 1992. Structure of the gene for human transglutaminase 1. J Biol Chem 267:17858–17863.

Yuspa SH, Harris CC. 1974. Altered differentiation of mouse epidermal cells treated with retinyl acetate in vitro. Exp Cell Res 86:95–105.