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The Genes Encoding Geranylgeranyl Transferase α-Subunit and Transglutaminase 1 Are Very Closely Linked but Not Functionally Related in Terminally Differentiating Keratinocytes

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We report here the entire exon/intron structure of the gene encoding the α-subunit of human Rab geranylgeranyl transferase (*RABGGTA*) gene, which is positioned in a tandem head-to-tail arrangement with the transglutaminase 1 (*TGM1*) gene, and its polyadenylation signal sequence is located just 2.3 kbp upstream of the capsite of *TGM1*. Even though *TGM1* and *RABGGTA* have different functions, their close localization raised the question as to whether they are functionally related in the epidermis. To address this question, we have studied the expression of the two genes by RT-PCR in normal human epidermal keratinocytes cultured under various differentiation conditions. While the expression of the *TGM1* gene is markedly affected by the calcium concentration of the medium, all trans retinoic acid, vitamin D_3, and TPA treatment, the expression of the *RABGGTA* gene was unaffected by these reagents. Taken together, even though these two genes are unusually closely linked, they are not functionally related in the terminal differentiation program of epidermal keratinocytes.

Transglutaminase 1 (TGase 1) is a member of the family of enzymes which catalyze the isopeptide crosslinking of proteins through the formation of N^{ϵ}-(γ-glutamyl) lysine bonds (1,2). TGase 1 is important for the formation of the cornified cell envelope, a 15-nm thick layer of insoluble protein deposited on the intracellular side of terminally differentiating stratified squamous epithelial cells. Mutations of the gene encoding the TGase 1 (*TGM1*), located at chromosome position 14q11.2 (3,4), are known to cause some cases of lamellar ichthyosis (5-8). In ongoing studies of the promoter region of human *TGM1*, previously we have cloned and sequenced a genomic clone containing 7.3 kb upstream sequences from the *TGM1* capsite (3).

Recently the complete cDNA sequence encoding the α-subunit of human Rab geranylgeranyl transferase (Rab GGTase) was reported (9), and remarkably, its distal 3′-sequences showed high homology to portions of a 2.9 kbp clone of the rabbit *TGM1* promoter (10). Sequence alignments suggested that the *RABGGTA* and *TGM1* genes may be very closely linked to each other, so that the former may overlap key regulatory elements of the latter (9). We have found that the 7.3 kbp of sequence of the human *TGM1* obtained in our laboratory covers most of coding region of the human *RABGGTA* gene, and in this paper we describe the complete gene structure of this gene.

The Rab GGTase enzyme (formerly known as component B or GGTase-II) attaches 20-carbon geranylgeranyl groups to Rab proteins that bear double cysteine motifs at their COOH-terminus such as Cys-X-Cys and Cys-Cys, where X may be any amino acid (11). Thus the TGase 1 and Rab GGTase enzymes have different functions. However, the observation that their genes are so closely organized in the genome and overlap if not share common regulatory elements may mean they are coexpressed and functionally related in keratinocytes (12), as for example are the cluster of genes encoding the epidermal differentiation complex on chromosome 1q21 (13). Furthermore, the close linkage raises the possibility that the *RABGGTA* gene may be involved in cutaneous disease. To investigate these questions, we have studied the expression of these two genes by RT-PCR in normal human epidermal keratinocytes (NHEK) cultured under various differentiation conditions.

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MATERIALS AND METHODS

Characterization of a human genomic clone containing the promoter region of TGM1. To characterize the promoter region of the *TGM1* gene, we have sequenced 7.3 kbp of the 5′-end of a human genomic clone gTGM1-32 (3). This data contained all but 383 nt of the cDNA sequence reported for the α-subunit of Rab GGTase enzyme (9). To complete the gene sequence we designed a primer from the 5′-end cDNA sequence of Rab GGTase enzyme α-subunit (1+: 5′-CCTCGCGCT-CTGGCCCGGGCAAT-3′), and a primer from 5′-end sequence of the gTGM1-32 clone (2-: 5′-CCAGCTGCTGGAGCA-CCTCTCGT-3′). By standard PCR procedures using human genomic DNA as template (4), we amplified a 1041 bp PCR product, which was sequenced to recover the missing genomic sequences.

Primer extension analysis. To confirm that the 5′-end of the cDNA sequence of the Rab GGTase α-subunit (9) included the entire transcript, we performed a primer extension experiment. An AMV reverse transcriptase primer extension system kit (Promega, Madison, WI) was used according to manufacturer's protocol. In brief, a specific backward primer (5′-GACTAGGACTTCCATCCGTGTG-3′) designed from the available 5′-non-coding sequences of *RABGGTA*, was end-labeled with γ-^{32}P-dATP, and annealed with 1 μg of polyA$^+$ RNA extracted from human fetal brain tissue (Clontech, Palo Alto, CA) at 60°C for 30 min followed by extension for 45 min at 42°C.

Cell culture. Cryopreserved NHEK cells were obtained from Clonetics (San Diego, CA) and grown in submerged cultures on calf skin collagen- (Sigma, St. Louis, MO) coated flasks in keratinocyte growth medium (Clonetics) in 0.05 mM calcium and 60 μg/ml bovine pituitary extract. Third passage cells were seeded at 5×10^3 cells per cm^2 and grown until they were 80% confluent. At this time the medium of some flasks was shifted to 1.2 mM calcium and maintained for up to 8 days. At the time of the calcium shift some cultures were grown in the presence of 1.2 mM calcium plus retinoic acid (5×10^{-7} M), or vitamin D_3 (1×10^{-7} M), or TPA (10 ng/ml), or DMSO (0.01%, as solvent control). Retinoic acid, vitamin D_3, TPA and DMSO were added to the high calcium media just before use.

RT-PCR. Cells from one or two 75 cm^2 culture flasks were used to isolate total RNA (15). RT-PCR reactions were performed using the EZ rTth RNA PCR kit (Perkin Elmer, Branchburg, NJ) following the manufacturer's instructions. The reaction conditions were carefully examined to stop the reaction during the exponential phase of amplification of each gene. Briefly 100 ng (for amplification of *RABGGTA* or *GAPDH*), 25 ng (for *TGM1*), or 0.4 ng (for 18*S* rRNA), of total RNA were reverse transcribed and amplified in the same tube in a total reaction volume of 10 μl in the presence of 3 μCi of α-^{32}P-dCTP (Amersham, Arlington Heights, IL). The amplification parameters were as follows: 2 min at 95°C; 45 sec at 95°C; and 45 sec at 60°C. Linear amplification was observed after 20 cycles (for *RABGGTA, GAPDH* or 18*S* rRNA) or 13 cycles (for *TGM1*). Five to ten μl of the reaction were electrophoresed on a 6% polyacrylamide gel, which was then dried. For quantitation of the RT-PCR products, bands were excised from the gel and counted on a scintillation counter Beckman LS6500. The primers used were: *TGM1:* (+) 5′-CCCGTACTGTCACCAACT-3′, (−) 5′-GGCCTCTTCATCCAGCA-GTC-3′; *RABGGTA:* (3+) 5′-TCAGACTTCAAGGGCTACCACTGG-3′, and (2-); *GAPDH:* (+) 5′-TGAAGGTCGGAGTCAACGGATTG-3′, (−) 5′-GCCATGGAATTTGCCATGGGTGG-3′; 18S rRNA (+) 5′-AGTTGCTGCAGTTAAAAAGC-3′, (−) 5′-CCTCAGTTCCGAA-AAC-CAAC-3′. Each primer was designed to cross over intron/exon boundaries to ensure amplification of mRNA only. Products were validated by size determination and restriction enzyme digestion

RESULTS AND DISCUSSION

Genomic organization of RABGGTA. We have previously described the human genomic clone gTGM1-32 which contains the first nine exons of and 7.3 kbp of upstream sequences of the *TGM1* gene (3). Following complete sequencing, this 7.3 kbp upstream region was found to contain 14 exons and 13 introns of the *RAB-GGTA* gene. However, comparisons with the published cDNA sequence encoding human Rab GGTase (9) revealed that 383 bp of cDNA sequences were missing from the 5′-end. By PCR, using primers located at the 5′-end of the published cDNA sequence and at the 5′-extent of our genomic clone (see Methods), we generated a 1041 bp fragment which encoded two additional exons and introns of *RABGGTA*. Exon I of the *RAB-GGTA* gene contained 272 nt of the 5′-non-coding region, including three nt of the likely initiation codon, and exon II contained the missing 111 nt of coding sequences (Fig. 1).

The complete genomic structure of the human *RAB-GGTA* gene consisted of 16 exons encoding 567 amino acids and 15 introns, and spanned 5858 bp. Fig. 2 shows the genomic organization of *RABGGTA* gene together with the intergenic sequence between *RAB-GGTA* and *TGM1*. The capsite of *TGM1* is located 2332 bp downstream from the end of the *RABGGTA* transcript so that the two genes are organized in a tandem head-to-tail arrangement. 5′-prime extension analysis (data not shown) revealed that the likely capsite of the *RABGGTA* gene is located within 10 bp of the published cDNA sequence. By use of a commercially available sequence comparison program, there was no significant homology in either exon or intron sequences between *RABGGTA* and *TGM1* (data not shown). Comparisons of our genomic sequences and the cDNA sequences (9) revealed two nt variations that resulted in amino acid changes: codon 404 (exon XII) AGG (arg) changed to ACC (thr); and codon 502 (exon XV) GAG (glu) changed to GAC (asp).

The RABGGTA and TGM1 genes are not co-regulated by calcium in NHEK cells. Because of the close proximity of the *RABGGTA* and *TGM1* genes, we wanted to investigate whether they are co-regulated during terminal differentiation in keratinocytes. We cultured NHEK cells and induced them to differentiate with calcium. Total RNA was extracted from cultures and used for RT-PCR to amplify *TGM1, RABG-GTA, GAPDH* and 18S rRNA. For each pair of primers, cycling parameters and reaction conditions were established to ensure that amplification remained in the linear range (see Methods).

Fig. 3A shows the results for *RABGGTA* and *TGM1* when normalized for *GAPDH* mRNA content. While *TGM1* mRNA expression showed an induction in cells grown in high calcium medium, *RABGGTA* mRNA expression was repressed. Since some of the total RNA samples exhibited consistent and significant discrepancies between optical density readings and *GAPDH* content, we repeated the experiments using 18*S* rRNA for

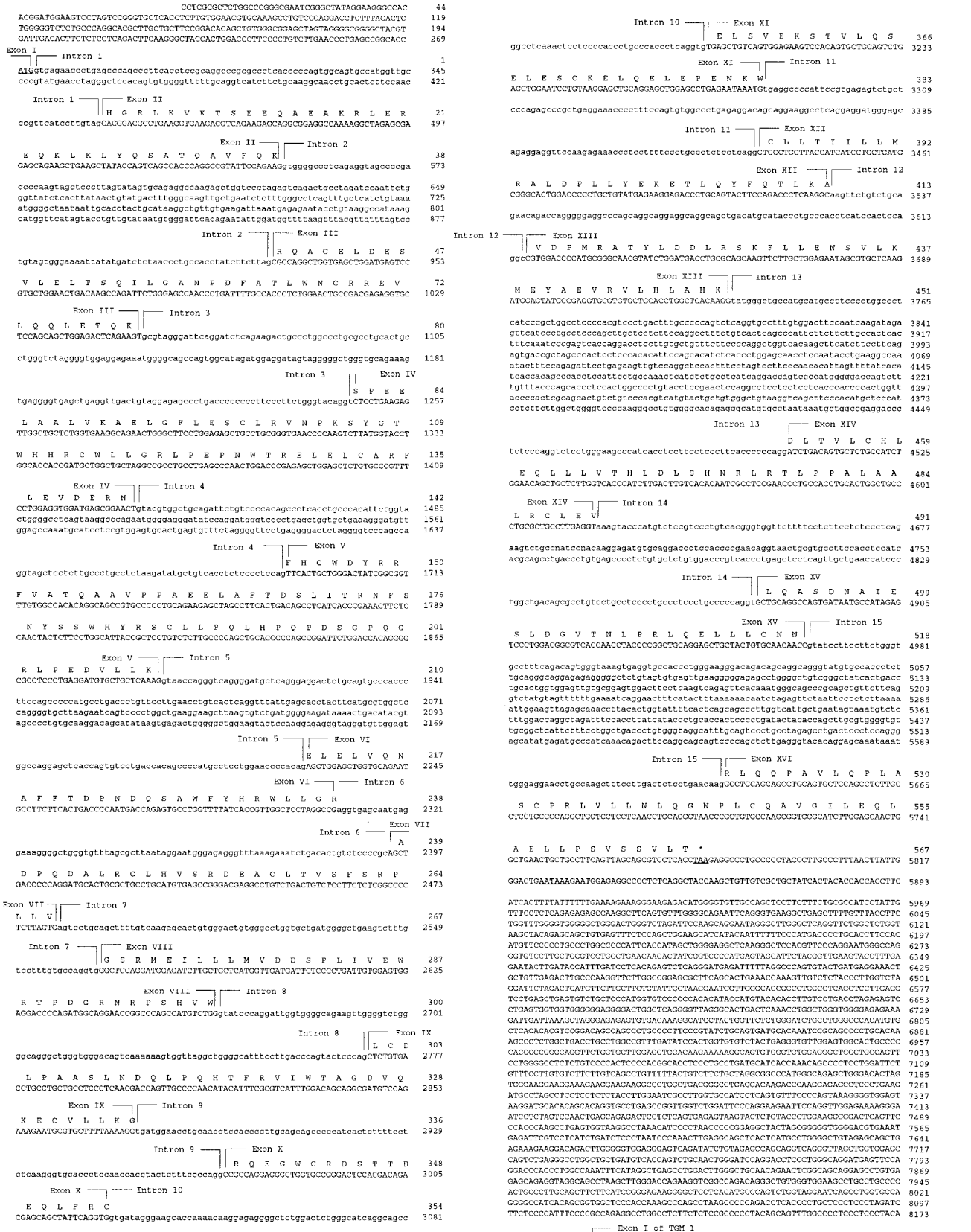

FIG. 1. Complete structure of the *RABGGTA* gene. Exon and intergenic sequences are shown in uppercase letters. The exon/intron boundaries are shown. The termination and polyadenylation signal sequences are underlined. The location of the capsite of the *TGM1* gene is shown.

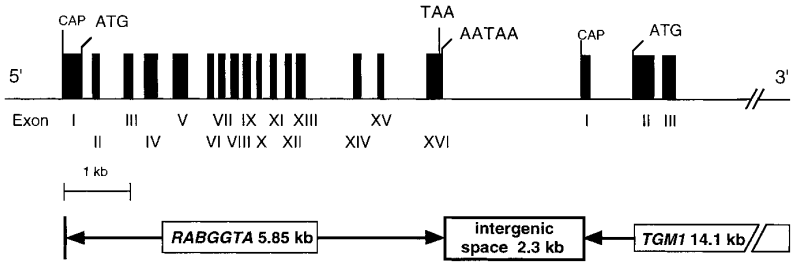

FIG. 2. Diagrammatic structure of the exons and introns of the *RABGGTA* gene and part of the *TGM1* gene.

normalization. 18*S* rRNA content was found to be in very good agreement with spectrophotometric measurements. Fig. 3B shows that *TGM1* was strongly induced by the calcium treatment, as expected (16-18).

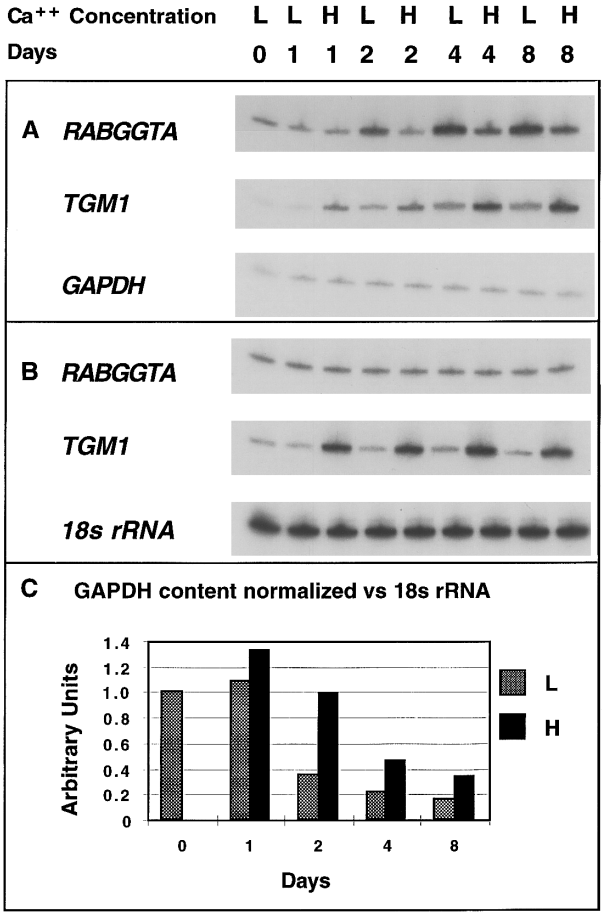

FIG. 3. Expression of *TGM1* and *RABGGTA* genes in low and high calcium media: difficulty of normalization. The *RABGGTA, TGM1,* and *GAPDH* mRNAs and 18*S* rRNA were examined by RT-PCR amplification. Total RNA was extracted from cultured NHEK cells grown in serum-free medium supplemented with 0.05 mM (L) or 1.2 mM (H) calcium (Ca^{2+}). Bands of amplified DNA were cut out and counted to obtain the illustrated quantitative data. (A) Results normalized to *GAPDH* mRNA content; (B) results normalized to 18*S* rRNA content; (C) *GAPDH* mRNA content normalized to 18*S* rRNA content.

However the *RABGGTA* mRNA was unchanged when the samples were normalized with respect to 18*S* rRNA content.

GAPDH is not a reliable standard for mRNA levels in NHEK cells. Thus it appears that although often used as a normalization control in RT-PCR reactions, *GAPDH* mRNA is not reliable in studies concerning differentiating keratinocytes. Fig. 3C shows that *GAPDH* mRNA expression in NHEK is reduced by a factor of 6-fold and 3-fold following achievement of confluency in low calcium and high calcium media, respectively. Hence normalization of gene expression in NHEK to *GAPDH* mRNA content leads to a several-fold overestimate and may lead to an incorrect conclusion. In the case of expression of the *TGM1* gene, in which induction by calcium is large (17), this discrepancy does not alter data interpretation, unlike in the case of the *RABGGTA* gene reported here. However, in the case of *TGM3* gene, calcium induced expression increases by only about 3-fold when normalized to 18*S* rRNA content (19,20; PMS, unpublished data) so that its induction properties might not be observed if *GAPDH* mRNA were used for normalization. Thus media calcium concentration and confluency (this paper) as well as UV light (21) and dioxin (22) affect *GAPDH* expression in keratinocytes. Therefore while *GAPDH* has been widely believed to be a constitutively expressed housekeeping gene, its variable expression in keratinocytes makes it unsuitable for the purpose of normalization.

RAGGGTA gene expression is not affected by modifiers of differentiation in NHEK cells. We further explored the expression of the *RABGGTA* gene in cultured NHEK treated with calcium and retinoic acid, vitamin D_3, or TPA. Fig. 4 shows that *TGM1* expression is upregulated by TPA at 1 day, downregulated by retinoic acid at all time points, and at 1 and 2 days by vitamin D_3, as reported previously (10,18,23,24). However, *RABGGTA* expression was unaffected by all treatments.

CONCLUSIONS

We describe the complete genomic structure of the human *RABBGTA* gene and show that it is only 2.3

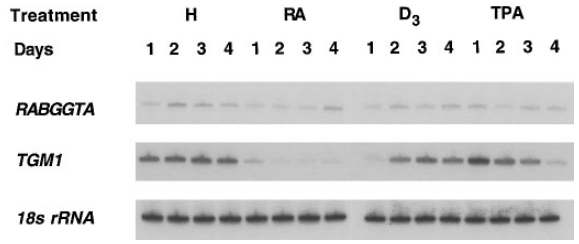

FIG. 4. The *RABGGTA* gene is constitutively expressed in keratinocytes and is not affected by modifiers of differentiation. RT-PCR amplification of *RABGGTA* and *TGM1* mRNAs was normalized to 18*S* rRNA content. Total RNA was extracted from cultured NHEK cells grown for the number of days as shown in serum-free medium supplemented with 1.2 mM Ca^{2+} (H) plus retinoic acid (RA) or vitamin D_3 (D_3) or TPA. These data are representative of 3-5 separate experiments.

kbp upstream from the *TGM1* capsite. Our expression data indicate that the *RABGGTA* gene is constitutively expressed in NHEK cells and is unaffected by conditions or reagents that affect differentiation in these cells. Thus, while the *RABGGTA* and *TGM1* genes are closely linked, overlap and possibly share some common regulatory elements (9), they are not co-regulated in the terminal differentiation program of cultured NHEK cells.

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