The transglutaminase I (TGase I) gene encodes an enzyme that catalyzes the cross-linking of structural proteins involved in the formation of the cornified envelope during squamous cell differentiation. To identify DNA elements important for the transcriptional control of the TGase I gene, we analyzed the ability of a 2.9-kilobase pair (kb) upstream regulatory region to control the expression of a reporter gene in vivo and in vitro. Transgenic mice bearing the pTG(--2.9kb)CAT construct exhibited the same pattern of tissue-specific expression of CAT as reported for TGase I. Deletion analysis in transiently transfected rabbit tracheal epithelial cells indicated that two sequences from bp −490 to −470 and from −54 to −37 are involved in the activation of TGase I transcription. Point mutation analysis and mobility shift assays showed that the sequence located between −54 and −37 is a functional Sp1-like transcription element. Sp1 and Sp3, but not Sp2, are part of nuclear protein complexes from differentiated RbTE cells binding to this site. The element TGATGTCGA between bp −490 and −470 is contained in a larger 22-bp palindrome and resembles the consensus cAMP response element-binding protein (CREB)/AP-1 element recognized by dimeric complexes of members of the CREB, ATF, Fos, and Jun families. Mutations in this sequence greatly reduced promoter activity. Supershift analysis identified CREB1, JunB, c-Fos, Fra-1, and c-Jun in protein complexes isolated from differentiated rabbit tracheal epithelial cells binding to this site. Our study shows that the Sp1- and CREB/AP-1-like sites act in concert to stimulate transcription of the TGase I gene. The 2.9-kb promoter region could guide expression of specific genes in the granular layer of the epidermis and could be useful in gene therapy.

Transglutaminases (TGases)1 (EC 2.3.2.13) constitute a family of Ca\(^2+\)-dependent enzymes that covalently cross-link proteins by catalyzing the formation of isopeptide bonds between the γ-amide group of glutamine and the ε-amino group of lysine (1–5). TGases have functions in a variety of biological processes, such as differentiation, apoptosis, and blood clotting (4–9), and are implicated in a number of diseases (4, 10–12). Factor XIII catalyzes the cross-linking of a number of proteins in plasma and plays an important role in fibrinogenesis (1, 2, 4, 5). Tissue TGase (TGase II) has been implicated in the activation of certain cytokines (13, 14) and cross-linking of specific components of the extracellular matrix (4). This TGase has also been reported to have GTPase activity and as such may have a role in signal transduction (15). In addition, evidence has been provided indicating that TGase II may catalyze cross-linking of intracellular proteins during apoptosis (8, 16). TGase I and III are involved in the formation of the cross-linked envelope during squamous cell differentiation (5–7,17–19).

Squamous differentiation is a terminal pathway of differentiation that occurs as a multistep process (21–23). In epidermal keratinocytes this differentiation represents a normal process, whereas in tracheobronchial epithelial cells, it is an aberrant pathway of differentiation that occurs under conditions of severe vitamin A deficiency or after injury (20–22). Early during differentiation, cells become committed to irreversible growth arrest that is accompanied by alterations in the expression of several growth-regulatory genes (6, 23–25). In subsequent stages in the differentiation process, cells begin to express a variety of squamous-specific genes (20–22). In tracheobronchial epithelial cells, squamous differentiation is associated with induction of keratin 13 (26, 27), relaxin (28), cholesterol sulfotransferase (29), epithelial membrane protein 1 (30), and several proteins involved in the formation of the cross-linked envelope (31–38).

The formation of the cross-linked envelope is a characteristic event in the final stages of squamous differentiation. The envelope consists of a layer of cross-linked protein deposited just beneath the plasma membrane (5–7, 18). It contributes to the cohesiveness and rigidity of the cornified layer and provides a vital barrier function. The envelope is formed by the cross-linking of many precursor proteins, including involucrin (38), cornifins/small proline-rich proteins (SPRRs) (32, 35, 36), and loricin (37) catalyzed by TGase I and III (5–7, 17–19). TGase I is synthesized as a soluble enzyme in the cytosol and becomes associated with the cell membrane after acylation by palmitic and myristic acid at a cluster of five cysteines at its amino terminus (39, 40). The TGase I gene has been cloned from various species (33, 41, 42). The human TGase I gene, which spans 14.3 kb and contains 15 exons, has been mapped to chromosome 14q11 (TGM1 locus) (43–45). The importance of this enzyme in the morphogenesis and function of the skin was recently corroborated by studies implicating mutations in the TGase I gene that result in a lack of TGase I activity in the...
Autosomal recessive skin disorder lamellar ichthyosis (11, 12). Recent studies demonstrated that TGase I-null mice exhibit severe deficiencies in several skin functions, including impairments in cell envelope assembly and barrier function (46).

Analysis of the TGase I promoter may provide insight not only into the molecular mechanisms controlling this gene in squamous epithelia and squamous metaplasia but also into the molecular pathogenesis of lamellar ichthyosis and lead to new methods of treatment, including drug and gene therapy. In this study, we analyzed the ability of a 2.9-kb promoter regulatory region of the TGase I gene to control the expression of a chloramphenical acetyltransferase (CAT) reporter gene in vivo and in vitro. We show that this region directs expression of the transgene to the suprabasal layers, comprising the late spinous and granular layers, of several squamous tissues. This pattern of expression correlates well with that reported for TGase I (47, 48).

Deletion analysis identified two DNA elements in the 2.9-kb regulatory region containing an Sp1- and a CREB/AP-1-like site that are involved in the transcriptional regulation of TGase I.

**Experimental Procedures**

**Cell Culture**—Rabbit tracheobronchial epithelial (RbTE) cells were cultured in Ham’s F-12 medium supplemented with 10 ng/ml epidermal growth factor, 5 μg/ml transferrin, and insulin as described previously (49). Normal human epidermal keratinocytes (NHEK) were purchased from Clonetics (San Diego, CA) and cultured in KGM medium (Clonetech). Rabbit tracheal fibroblasts were grown in Ham’s F-12/RPMI 1640 from Clonetics (San Diego, CA) and cultured in KGM medium (Clonetics). Rabbit tracheal fibroblasts were grown in Ham’s F-12/RPMI 1640 from Clonetics (San Diego, CA) and cultured in KGM medium (Clonetics). Rabbit tracheal fibroblasts were grown in Ham’s F-12/RPMI 1640 from Clonetics (San Diego, CA) and cultured in KGM medium (Clonetics).

**Plasmids and Reporter Constructs**—The plasmid pTG(-2.9kb)-LUC containing the luciferase reporter gene under the control of the -2.9 kb promoter flanking region of the TGase I gene has been previously described (34). The deletion mutants pTG(-1.5kb)-LUC and pTG(-350)-LUC were constructed using the Sma1 and Apa1 restriction sites (Fig. 1A). The minimal promoter construct pTG(-37)-LUC and pTG(-54)-LUC were made by cloning the respective PCR products into the promoterless luciferase vector pGL2-Basic (Promega). Deletion mutants in region -750 to -350 bp were constructed by PCR-directed cloning into the construct pTG(-37)-LUC (Fig. 2A). All PCR reactions were conducted with proofreading Vent DNA polymerase (New England Biolabs), and the original 2.9-kb construct was used as template. All primers contained a six-base 5’-leader sequence followed by a six-base restriction site (KpnI, MluI, or XhoI) and a 20-base promoter sequence. Point mutation constructs were made by direct cloning of chemically synthesized oligonucleotides upstream of pTG(-37)-LUC. The sequences of the PCR products were checked for accuracy using the dideoxynucleotide chain termination method and the Sequenase Quick-denuature plasmid sequencing kit (Amersham Pharmacia Biotech).

**Search for Transcription Factor Binding Sites**—Promoter flanking regions were analyzed for the presence of potential transcription factor binding sites using the TFSEARCH Program.²

**Transfections and Reporter Gene Assays**—Plasmid DNA used in transfections were isolated and purified with a Wizard Miniprep kit (Promega). RbTE cells were grown to confluence in six-well dishes, and transfections were carried out in triplicate using lipofectamine (Life Technologies, Inc.) according to the manufacturer’s protocol. Cells were incubated in 1.0 ml of Ham’s F-12 medium without growth factors and antibiotics in the presence of 1.5 μg of total DNA and 5 μl of lipofectamine. Cotransfection with β-actin-CAT reporter plasmid was carried out to correct for differences in transfection efficiency. After a 5-h incubation, medium was replaced with complete F-12 or KGM medium. Cells were collected 48 h after transfection and assayed for reporter activity. Whole cell extracts were prepared with the lysis buffer included in the CAT ELISA kit (Boehringer Mannheim) according to the manufacturer’s protocol and used in CAT and luciferase assays. Luciferase assays were performed with the Luciferase Assay Kit from Promega. The relative luciferase activity was normalized for CAT reporter activity.

**Electrophoretic Mobility Shift Assays (EMSAs)**—Nuclei were isolated from differentiated rabbit tracheobronchial epithelial cells, and nuclear extracts were prepared by the method of Dignam et al. (50) with slight modifications. Briefly, cells were washed twice in ice-cold PBS, collected into microtubes, and centrifuged for 10 s in a microcentrifuge. The cell pellet was then resuspended in 1 ml of ice-cold, low salt buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM EGTA, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 μg/ml leupeptin and aprotinin, 0.1 mM sodium vanadate). After incubation on ice for 20 min, Nonidet P-40 was added to a final concentration of 0.5%. The cell suspension was vigorously vortexed for 20 s followed by centrifugation for 30 s in a microcentrifuge to collect the nuclei. The nuclei were resuspended in 10 volumes of high salt buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 0.42 M NaCl, 10% glycerol, and protease inhibitors) and incubated on ice for 20 min followed by a 10-min centrifugation in a microcentrifuge at 4 °C. Nuclear extracts were collected and stored in aliquots at -70 °C. The Sp1 consensus oligonucleotide (CTCCGCCCCGCCGCCAGT-GAAT), the AP-1 consensus (CGGTGATTGAGTCAGCCGGAAA), the

²This program is available on the World Wide Web at http://poppet.trec.wcrp.or.jp/research/db/TFSEARCH.html.
CREB consensus (GTACTGGCTGTGACGTCACCAGCCA), and other oligonucleotides were synthesized using a model 392 DNA synthesizer (Applied Biosystems). Oligonucleotides for EMSA were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (Promega) and purified with NAP-5 columns (Amersham Pharmacia Biotech). Approximatly 1 ng (50,000 cpmp) of the oligonucleotide probe and 0.5 μg of nuclear extract were used in the binding reaction in buffer containing 20 mM HEPEs, pH 7.9, 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol. To prevent nonspecific binding, 1 μg of poly(dI-dC) and 0.2 μg of salmon sperm DNA were included in the reaction. The binding reactions were carried out at room temperature for 2 h, and the presence or absence of a 10- or 100-fold molar excess of oligonucleotide competitors. For supershift analysis, various antibodies were included in the incubation mixture. DNA-protein complexes were separated on 5% nondenaturing polyacrylamide gel electrophoresis at 200 V for 1.5 h. The DNA bands were visualized by autoradiography.

Footprint Analysis—A TGase I promoter fragment from −750 to −350 bp was generated by PCR with proofreading Vent DNA polymerase (NE Biolabs). One of the primers used for PCR was labeled with T4 polynucleotide kinase (Promega). The binding mixture contained 20 μM HEPES (pH 8.0), 10% glycerol, 50 mM NaCl, 10 mM MgCl2, 10–20 μg of nuclear extracts, 1 μg of labeled DNA, and 1 μg of poly(dI-dC). The binding reaction was carried out for 25 min at room temperature and then incubated with different concentrations of DNase I. After 1 min of incubation with DNase I, 1 volume of stop buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% SDS) was added. DNA was purified by phenol extraction and concentrated with Microcon-30 (Amicon). DNA fragments were separated on a 6% sequencing gel.

Generation and Identification of Transgenic Mice—The construction of the pTG(−2.9)-CAT reporter was carried out by cloning the 2.9-kb promoter fragment (KpnI/NhelI restriction fragment) from pTG(−2.9)LUC into the XhoI site of the promoterless pCAT reporter plasmid (Promega). Compatible end ligation of the Nhel site into the XhoI site was followed by incubation with T4 DNA polymerase (Amersham Pharmacia Biotech). The resulting blunt ended XhoI and KpnI sites were then autoligated. Orientation of the clones was confirmed by sequencing. This plasmid was linearized, and a 4.5-kb HindIII/BamHI DNA fragment containing the −2873 to +89 bp regulatory region of TGase I gene and the CAT reporter excised. The fragment was purified and microinjected into the pronuclei of one-cell mouse embryos obtained from C57BL/6J females mated with SJL F2 males. The embryos were then transferred to the oviduct of pseudopregnant females, and normal gestation was allowed. Positive founder transgenic mice carrying the construct were identified by PCR analysis of genomic DNA isolated from tail biopsies. A rabbit TGase I promoter-specific primer, 5'-TCGGGCCCCCTCCCGCA, and a CAT-specific primer, 5'-AACGGTGGTTATATCCAGTGTA, were used for the analysis. Tissues for CAT assay, RNA isolation, and in situ hybridization were taken from 3- or 12-month-old mice.

In Situ Hybridization—Transgenic mice were sacrificed, and tissues isolated and fixed for 3 h with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4. After rinsing in phosphate-buffered saline, tissues were dehydrated in a graded ethanol series, permeated with xylene, and embedded in paraplast. In situ hybridization was performed as described (51) with the following minor modification. Serial sections (5 μm) were mounted on Silane-prep® slides (Slide), deparaffinized, acetylated, and pretreated with 2× sodium citrate (SCC), 50% formamide, 10 mM diithiothreitol at 50°C. Radiolabeled sense and antisense CAT probes were generated with either T3 or T7 RNA polymerase and [35S]-labeled UTP and CTP (Amersham Pharmacia Biotech; 1000 Ci/mmoll, as described (51)). Probes were subjected to limited alkaline hydrolysis to reduce the size of the transcripts to about 150 bases. After hybridization at 50°C for 16 h, the sections were washed and treated with RNase. The slides were dipped in NTB-2 emulsion (Eastman Kodak Co.) diluted 1:1.5 and exposed for 14 days at 4°C. Slides were developed and counterstained with hematoxylin.

RESULTS

A 2.9-kb TGase I Promoter Region Is Sufficient for Tissue- and Differentiation-specific Expression—The cloning and sequencing of the 2.9-kb 5′-flanking region of the rabbit TGase I gene has been reported previously (26). We also mapped the transcription start site and showed that this transgenic region was able to induce transactivation of the luciferase reporter gene during differentiation of cultured RbTE cells (26). To determine whether this region is able to control the expression of TGase I in vivo in a cell type- and differentiation-specific manner, we constructed an expression vector in which the CAT gene has been reported previously (26). We also mapped the transcription start site and showed that this transgenic region was able to induce transactivation of the luciferase reporter gene during differentiation of cultured RbTE cells (26). To determine whether this region is able to control the expression of TGase I in vivo in a cell type- and differentiation-specific manner, we constructed an expression vector in which the CAT reporter is under the control of the 2.9-kb 5′-flanking region of the rabbit TGase I gene and introduced this plasmid into the mouse germ line. The presence of this transgene was corroborated by PCR. Two independent founders, which contained and expressed the integrated transgene, were selected for subsequent analysis. Protein extracts prepared from various tissues of control and transgenic mice were analyzed for CAT protein concentration. As expected, no CAT protein was detectable in any of the tissues isolated from control mice (Fig. 1B). In the transgenic mice, CAT protein was only detected in crude protein extracts from tissues that normally express TGase I, including skin, tongue, and esophagus. The highest CAT levels were found in extracts from tongue and esophagus; lower levels were found in skin. This pattern of CAT expression is consistent with the known expression levels of TGase I in these tissues (34). These results demonstrate that this 2.9-kb 5′-flanking region of the TGase I gene contains regulatory elements that are sufficient to control the transcription of this gene in a tissue-specific manner.

During squamous differentiation, cells transit from the basal into the suprabasal layers of the epithelium and begin to express a variety of differentiation-specific genes. Initiation of TGase I expression occurs within the late spinous and granular cell layers of the squamous epithelium. To localize the expression of the transgene in squamous tissues, we performed in situ hybridization using sense and antisense riboprobes to the CAT mRNA. A specific hybridization signal was observed in the suprabasal layers, in particular in the late spinous and granular layers of the squamous epithelium of the tongue from the transgenic mice, as shown in Fig. 2, A and B. The hybridization signal in the basal layer was at background levels. A low background hybridization signal was observed in squamous epithelia of control mice with no significant differences between the different layers (Fig. 2, C and D). These results indicate that the 2.9-kb region contains DNA elements that are able to regulate transcription of CAT at a specific stage during
differentiation that is similar to the pattern of endogenous TGase I expression (47).

Deletion Analysis of the TGase I Promoter—In an effort to locate regulatory sequences important in the transcriptional control of the TGase I gene, a series of deletion constructs were made using several unique restriction sites within the 5' flanking region or fragments generated by PCR (Fig. 3A). Transcriptional activity of these pGL2-LUC constructs was analyzed by transient transfection into primary rabbit tracheobronchial cells cultured under differentiation-inducing conditions. Compared with the activity induced by the minimal promoter (bp 237 to 160), the full-length 2.9-kb region was able to increase luciferase activity by about 30–50-fold, depending on the experiment. Deletions up to bp −750 had only a small effect (10% reduction) on luciferase activity, indicating that the region between bp −2874 and −750 does not contribute significantly to differentiation-specific activation when transfected into squamous-differentiated RbTE cells (Fig. 3). In contrast, deletion of the region spanning bp −750 to −348 resulted in a 70% decrease in luciferase activity. Further deletions of the region up to bp −54 had only minor effects; however, deletion of the region between bp −54 and −37 caused an additional 90% reduction in promoter activity (Fig. 3B). As demonstrated in Fig. 3C, the minimal TGase I promoter (pTG(−37/+60)LUC) containing the TATA box-like sequence CATTTAA still exhibited transactivating activity compared with the promoterless pGL2-LUC. Deletion of this sequence (pTG(0/+60bp)LUC) or the region downstream of CATTTAA (pTG(−37/0)LUC) completely abolished the ability to activate transcription (Fig. 3C).

Analysis of the promoter activity of TGase I deletion constructs in differentiated NHEK showed that the relative transcriptional activation of the reporter gene by the various pro-
Transcriptional Regulation of Transglutaminase I

The Proximal TGase I Promoter Contains a Functional Sp1 Element—The proximal region was analyzed with TFSearch for the presence of potential transcriptional factor binding sites. This computer search indicated that the −54/−37 region responsible for 5–10-fold transactivation of the basal TGase I promoter contains an Sp1 consensus sequence at −47 to −40 (Fig. 5A). A two-base mutation in this Sp1 element reduced transcriptional activity of the −54/+60 fragment to that of the minimal promoter (−37/−60), indicating that the region is necessary for transactivation. To map the exact position of the enhancer element within the TGase I promoter, we performed DNase I footprint analysis. The −2.9 kb region of the TGase I promoter, nuclear extracts from squamous differentiated RbTE cells, revealed the formation of several specific DNA-protein complexes. Unlabeled TG-Sp1 was able to compete for these complexes, while the mutated oligonucleotide TG-Sp1 competed much less efficiently (Fig. 5C). A commercially available Sp1 consensus oligonucleotide (Sp1cons) also competed well for binding. An antibody against Sp1 protein caused a supershift of the upper band, indicating that Sp1 is a major part of this protein complex (Fig. 5D). The second and third faster migrating bands were supershifted by antibodies against Sp3. Antibodies against Sp2 or Egr-1 (not shown), a transcription factor that binds the consensus sequence GCCGGGGCGG, failed to interact with any of the protein complexes binding TG-Sp1.

Deletion Analysis of −750/−350 Region of the TGase I Promoter—To identify elements in the −750/−350 region that are involved in the regulation of the TGase I gene, a series of overlapping deletion mutants was constructed by inserting various segments of this regulatory region into pTG(−37)-LUC (Fig. 6A). Transient transfection analysis showed that the region most important for transactivation was located between bp −490 and −470 (Fig. 6B). Deletion of this sequence within the bp −750 to −350 fragment decreased transactivation by more than 80%. When ligated to the minimal TGase I promoter (D10; Fig. 6B) this fragment increased the level of reporter gene activity by more than 10-fold. The −490 to −470 sequence consists of the almost perfect palindromic structure CTGGGTCGCTGAGCCAGCACAG (referred to as TGpal) containing a CREB/AP-1-like sequence (TGATGTCA) in the middle (Fig. 6C). It is interesting to note that this palindromic region including the CREB/AP-1-like element is highly conserved in the corresponding 5′-flanking region of the human TGase I gene (Fig. 6C).

Fig. 7 shows that the CREB/AP-1-like element (TGpal) and the Sp1 site are acting in concert to optimally increase transactivation. Each element used separately with the minimal promoter increased transactivation of the reporter, while together (TGpal/Sp1) they synergistically enhanced transactivation to a level slightly less than that of the full 2.9-kb 5′-flanking region.

To directly map protein binding sites in the −750/−350 region of the TGase I promoter, nuclear extracts from squamous differentiated RbTE cells and a 400-bp DNA fragment (−750/−350) were used for DNase I footprint analysis. Although several DNA elements were found to be protected, the region between bp −490 and −470 containing the TGpal was one of the most strongly protected sequences (Fig. 8).

TGase I Promoter Contains a Functional CREB/AP-1-like Element—To determine which bases in the TGpal element are optimal for its transactivation function, mutated oligonucleotides M1–M8 (Fig. 9A) were inserted upstream of the −37/+60 basic promoter region of pTG(−37)-LUC. The transcriptional activity of these constructs was tested in differentiating RbTE cells by transient transfection assays. As demonstrated in Fig. 9B, transactivation of the LUC reporter under the control of TGpal was increased about 10-fold compared with the activity of the basic promoter. The mutations (M2–M7) that disrupt transcriptional activation most are within the CREB/AP-1-like sequence (TGATGTCA).

To map the exact position of the enhancer element within the −490/−470 region of the TGase I promoter, we performed EMSA with a 32P-labeled TGpal oligonucleotide and nuclear extracts isolated from differentiating RbTE. Several oligonucleotides (M1–M8; Fig. 9A), containing different point mutations in this region, were tested for their ability to compete with 32P-labeled TGpal for protein binding. The oligonucleotide M1 competed as effectively as the unlabeled TGpal itself (Fig. 10A). The oligonucleotides M2–M4 and M8 were less effective competitors than unlabeled TGpal while M6, M7, and to a lesser degree M5 did not compete well for binding. These results indicate that TCA within the CREB-like element is optimal for the formation of DNA-protein complexes. Since TGpal contains an element that shows similarity to CREB and AP-1 sites, we
assessed the ability of consensus AP-1 (TGAGTCA) and CREB (TGACGTCA) oligonucleotides to compete with 32P-labeled TGpal for binding to nuclear proteins from squamous differentiated RbTE cells. As shown in Fig. 10B, both the consensus AP-1 and CREB oligonucleotides were able to compete with TGpal; however, they were less efficient than TG pal itself.

To determine the identity of some of the proteins in the complexes binding to TGpal, supershift analysis was carried out with nuclear extracts from squamous differentiated RbTE cells and antibodies against various members of the Jun, Fos, CREB, and ATF families of transcription factors. These analyses identified CREB1, c-Jun Fra-1, and c-Fos as part of protein complexes bound to TGpal; however, they were less efficient than TG pal itself.

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DISCUSSION

In this study, we analyzed the promoter activity of the 2.9-kb 5'-flanking region of the rabbit TGase I promoter in vivo and in vitro. Analysis of the TG(-2.9)-CAT transgenic mice revealed that CAT mRNA was only expressed in squamous tissues and was restricted to the suprabasal layers comprising the late spinous and granular cell layers. This pattern of transgene expression correlates well with that reported for endogenous TGase I mRNA (47). These results demonstrate that this 2.9-kb region contains elements that are sufficient to direct the tissue-
and differentiation-specific expression of TGase I in vivo and are in agreement with a recent report on the promoter activity of the human TGase I in transgenic mice (54). This conclusion is supported by observations in cultured cells showing that the 2.9-kb region of the TGase I promoter is able to control the transcription of a reporter gene in a cell type- and differentiation-specific manner (Fig. 4; Refs. 34 and 52).

In order to locate the regulatory elements required to drive TGase I expression during squamous differentiation, a series of deletions were made to the 2.9-kb 5'-flanking sequence. This analysis mapped the minimal promoter to −37/+60 and identified two major sites that are important in the transcriptional control of TGase I: a palindromic sequence from −490 to −470 bp (TGpal) containing a CREB/AP-1-like site in the middle and the region from bp −53 to −37 consisting of a functional Sp1 site. We showed that these two elements work in concert to regulate the basal promoter activity of the TGase I gene. Previous studies implicated several AP-2-like sites in the transcriptional regulation of human TGase I in NHEK cells (51); these sites may cooperate with the Sp1 and TGpal elements.

The GC-rich region containing the Sp1 consensus element is located just upstream from the TATA-like box. Point mutations in this element reduce promoter activity to that of the minimal TGase I promoter (Fig. 5). Although another consensus Sp1 sequence is present at bp −194, no evidence was obtained indicating that this site is important in the regulation of the TGase I gene. Sp1 elements have been reported to be able to bind a number of transcriptional factors including members of the Sp1 family (56). Sp1 contains a DNA-binding domain consisting of three zinc fingers and binds the consensus sequence 5'-GGGCGG. In addition to Sp1, three related genes (Sp2, -3, and -4) have been identified (56, 57). These transcription factors can act alone or in cooperation with other transcription factors and can enhance or repress transcription depending on promoter context and cell type. Sp1 elements have been linked to the regulation of several genes during squamous differentiation. The Sp1 site is needed for optimal transcriptional activation of the involucrin gene, and protein complexes bound to this site were found to contain Sp1 but not Sp2, -3, or -4 (57). Sp1 sites have also been implicated in the regulation of SPRR-2A (59) and TGase III (60). Our mobility shift analysis demonstrated that in differentiated RbTE cells, Sp1 and to a
minor extent Sp3 are part of protein complexes that interact with the TG-Sp1 element. A recent study has demonstrated that the ratio of Sp1 to Sp3 is low in undifferentiated and high in squamous-differentiated NHEK cells and that Sp1 is involved in the differentiation-specific regulation of HPV-16 genes (61). This differential expression may hint at a possible role for Sp1-Sp3 antagonism during squamous differentiation.

Antagonism of Sp1-mediated transcriptional activation by Sp3 has been demonstrated in several other cell systems (56). In contrast to these observations, the differentiation-dependent, transient induction of the cyclin-dependent kinase inhibitor p21WAF1/Cip1 during early stages of squamous differentiation in NHEK cells has been reported to depend on the interaction of Sp3 rather than Sp1 with a GC-box in the proximal promoter region of p21WAF1/Cip1 (62). The reported increase in the Sp1/Sp3 ratio during differentiation may be a later event and be part of the down-regulation of p21WAF1/Cip1. Moreover, the activation or repressor function of Sp3 may be determined by the sequence or context of the GC-box.

The functionally important CREB/AP-1-like element TGATGTCA is contained in a 22-bp palindrome that is protected in DNase I footprinting assays. Point mutations in the CREB/AP-1-like site reduced promoter activity of this site, supporting the importance of the CREB/AP-1 motif. The CREB/AP-1-like element has been shown to bind a variety of transcriptional factors, including members of the CREB, ATF, and Fos/Jun families (63). Mobility shift assays using an array of antibodies against different CREB/AP-1-binding proteins demonstrated that TGpal interacts with protein complexes containing CREB1, c-Jun, JunB, Fra-1, and c-Fos in nuclear extracts from differentiated RBTE cells. CREB/AP-1-like elements have been implicated in the transcriptional regulation of several other squamous marker genes, including involucrin, profilaggrin, and SPRR-1 genes (64–67). It is likely that the regulation of squamous-specific genes, which are induced at different stages during differentiation, involves control by different CREB/AP-1 protein complexes. The differential expression of members of the Jun/Fos family during different stages of squamous differentiation is in agreement with this concept (68). A role for CREB/AP-1-like elements is further supported by studies showing that PKC activation, which regulates the expression of Jun/Fos family members, is an important component of the signaling pathways controlling squamous differentiation and
TGase I (34, 68–71). Cholesterol sulfate, which is highly induced during squamous differentiation (71), can function as an endogenous second messenger and through the activation of different PKC isoforms enhance the expression of several squamous-specific genes, including TGase I (52, 70).

The role of specific CREB/AP-1 complexes in squamous differentiation is not yet well understood. Recent studies have demonstrated that expression of c-Fos appears not to be essential for squamous differentiation in the epidermis, since epidermal differentiation is normal in c-Fos null mice (68). Others have suggested that JunB may be involved in the induction of early markers, including involucrin and TGase I, while JunD may play a role in the control of later markers, such as profilaggrin (65). A role for JunB in TGase I regulation is supported by immunohistochemical studies showing that JunB is predominantly present in the granular layer where TGase I is also expressed (47, 68) and by EMSA demonstrating the presence of JunB in protein complexes binding TGpal. It is clear that multiple protein complexes in squamous differentiated cells can compete for binding to TGpal. Some of these complexes may act as repressors, while others may function as stimulators of gene transcription. Therefore, increased transcription of squamous-specific genes may depend on the level and activity of these complexes during the differentiation process. This may involve changes in heterodimerization partners that could alter the affinity and activity of the complex. This concept is supported by recent findings showing that ectopic expression of a dominant-active c-Jun has a repressive effect on the expression of several squamous-specific genes, including involucrin and SPRR-1 (73). In addition, it was demonstrated that c-Jun is expressed predominantly in basal and early spinous layer and that the level of c-Jun and phosphorylated c-Jun is dramatically down-regulated during squamous differentiation and is probably related to the observed reduction in JNK activity (68). These findings support a negative regulatory role of c-Jun in the control of squamous differentiation. The latter is in agreement with observations showing that squamous cell markers are not induced in squamous cell carcinoma cell lines overexpressing c-Jun (73).

In this study, we show that the 2.9-kb 5′-flanking region of the TGase I gene can regulate this gene in a tissue- and differentiation-specific manner and identify two DNA elements important in its transcriptional control. It is known that certain forms of lamellar ichthyosis are linked to defects in the expression of the TGase I gene (11, 12). In some cases, this genetically heterogeneous disease might be caused by mutations in transcriptionally important promoter regions of TGase I. Therefore, the study of this promoter region could help in understanding the nature of this disease and provide a tool for gene therapy of lamellar ichthyosis and other skin disorders.

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