Regulation of the *Dlx3* Homeobox Gene upon Differentiation of Mouse Keratinocytes*

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The *Distal-less Dlx3* homeodomain gene is expressed in terminally differentiated murine epidermal cells, and there is evidence to support an essential role as a transcriptional regulator of the terminal differentiation process in these cells. In an attempt to determine the factors that induce *Dlx3* gene expression, we have cloned the 1.2-kilobase pair proximal region of murine gene and analyzed its cis-regulatory elements and potential trans-acting factors. The proximal region of the *Dlx3* gene has a canonical TATA box and CCAAT box, and the transcription start site was located 205 base pairs upstream from the initiation of translation site. Serial deletion analysis showed that the region between −84 and −34 confers the maximal promoter activity both in undifferentiated and differentiated primary mouse keratinocytes. Gel retardation assays and mutational analysis demonstrated that the transcriptional regulator NF-Y (also referred to as CBF) binds to a CCAAT box motif within this region and is responsible for the majority of the *Dlx3* promoter activity. In addition, an Sp1-binding site was located immediately upstream of transcription start site that acts as a positive regulatory element of the *Dlx3* promoter, independent of the CCAAT box motif. Importantly, elements residing between +30 to +60 of the *Dlx3* gene are responsible for the Ca2+-dependent induction of *Dlx3* during keratinocyte differentiation.

During epidermal differentiation, mitotically active basal keratinocytes cease to proliferate, detach from the basement membrane, and migrate through the spinous and granular layers to the outermost terminally differentiated cornified layer of the skin. This cornification process is tightly associated with a stepwise program of transcriptional regulation and is concurrent with the sequential induction and repression of structural and enzymatic differentiation-specific markers (1). This process can be achieved in mouse keratinocytes cultured *in vitro* by increasing the Ca2+ concentration from 0.05 to 0.12 mM in the culture medium (2), which produces a situation that mimics the endogenous Ca2+ gradient present in the skin (3). The Ca2+ signaling differentiation pathway is associated with increased phospholipase C activity (4) and activation of protein kinase C (PKC) (5). Previous work has demonstrated an essential role of PKC signaling in the late stages of epidermal differentiation. Activation of PKC has been shown to be necessary for expression of late differentiation markers loricrin and profilaggrin and for the suppression of the spinous-specific markers K1 and K10 (6).

*Dlx3*, a murine ortholog of the *Drosophila Distal-less* homeodomain protein (7), is a member of the *Dlx* vertebrate family. This family comprises to date six genes identified both in mouse and human and found to be organized as three convergently transcribed pairs, each closely linked to one of the four mammalian Hox clusters (8–13). Disruption of the *DLX3* coding sequence has been associated with a human disorder, Treacher-Collins syndrome. This inherited autosomal dominant disorder is characterized by defects in ectodermal derivatives such as hair and teeth and craniofacial bone abnormalities (14). *Dlx3* is expressed in the granular layer of the epidermis and in the hair matrix cells of the hair follicle (15, 16), and there is evidence strongly supporting the critical role of the Dlx3 homeoprotein in the regulation of expression of late epidermal differentiation genes (15). In *in vitro* studies have shown that Dlx3 binds to an AT-rich region and acts as a positive transcriptional regulator (17), which is activated during the Ca2+ shift in keratinocytes induced to differentiate in culture (18). In transgenic mice, ectopic expression of *Dlx3* in basal cells is accompanied by the cessation of cell proliferation and the up-regulation of expression of late epidermal differentiation structural genes including profilaggrin (15). A potential binding site for Dlx3 has been identified in the profilaggrin gene, suggesting that the observed up-regulation of this gene in the transgenic mice may result from a direct effect of Dlx3 (15). Altogether, these data strongly support a role for Dlx3 as a determinant factor in the activation of expression of granular markers during the terminal differentiation of keratinocytes.

During the process of terminal epidermal differentiation, many genes expressed in the keratinocyte are regulated at the transcriptional level (1). The transcription factors AP1 and AP2 have been characterized as primary regulatory factors of keratinocyte gene expression (19–24). PKC is an upstream component of the pathway that regulates AP1 in many systems and may play a role in the epidermal differentiation expression of K5, K1, loricin, profilaggrin, and involucrin (19, 21–24). Members of the POU family of transcription factors such as Oct1, Oct2, Oct6, Skn1a, and Skn1i have also been implicated as regulators of epidermal genes (25–28).

In order to elucidate the role of Dlx3 in the cascade of transcriptional events that ultimately leads to terminal differentiation, we have cloned and characterized the mouse *Dlx3* promoter. Deletional promoter analysis was utilized to delineate the sequences that regulate the transcription of *Dlx3* in differentiated and undifferentiated keratinocytes. In turn, these cis-phenolic acetyltransferase; PCR, polymerase chain reaction; bp, base pair.

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1 The abbreviations used are: PKC, protein kinase C; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; bp, base pair.

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acting elements were used to identify the transcription factors that regulate the Dlx3 promoter. Importantly, we have identified a region residing between +10 and +60 that responds to the Ca2+ shift used to differentiate the keratinocytes in culture.

MATERIALS AND METHODS

Cloning of Upstream Regulatory Region of the Mouse Dlx3 Gene—To clone the upstream sequence of the Dlx3 gene we used the Genome-Walker Kit (CLONTECH). It is designed to amplify a specific genomic DNA using an adapter sequence attached to the 5'-end of the genomic DNA. The amplified DNA sequences were made by PCR using oligonucleotides containing each mutant site cloned DNA. The 5'-end of the oligonucleotides 10F (5'-acGTCGCTGCGTGCCTC-3') and 10R (5'-ctaCTGCGTGCCTC-3') were constructed using two oligonucleotides 10I (5'-cgctctagaTCTCCGTGTCCCAAGCCACAG-3') and 10J (5'-cgctctagaAGTCAAATGCTGCCAGCTCCGCCCGGCCAGCCG-3'). The steps of reverse transcription, tailing, and PCR were performed using the 5'-rapid amplification of cDNA ends system (Life Technologies, Inc.). The gene-specific oligomer was located at +124 bp from the translation initiation site of Dlx3. The final PCR products were cloned and sequenced to determine the extreme 5' mRNA sequence.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from undifferentiated and differentiated primary mouse keratinocyte cultures as described by Andrews and Fallier (30). All steps were carried out at 4 °C. The cells (1 × 10^6) were harvested, washed three times with ice-cold phosphate-buffered saline, and pelleted. The keratinocytes were resuspended in 0.4 ml of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 15 min, and then homogenized with a Dounce homogenizer B pestle for about 20–25 strokes. The homogenate was centrifuged at 14,000 rpm, and the nuclear pellet was resuspended in 0.1 ml of buffer B (50 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. After centrifugation at 14,000 rpm for 20 min at 4 °C, the supernatant fraction was aliquoted and stored at −70 °C. The protein concentration was determined using the Bradford assay following the methods described for the Bio-Rad Protein Assay.

Gel Retardation Analysis—Gel retardation analysis was carried out according to Ausubel et al. (31).32P-End-labeled DNA fragments (about 1 ng of DNA) were incubated for 15 min at 4 °C with nuclear extracts in a total volume of 15 μl of binding buffer (10 mM HEPES, pH 7.9, 60 mM KCl, 1 mM EDTA, 10% glycerol, 10 mM MgCl2 containing 2 μg of poly[d(I-C)]). The products of the DNA-protein binding reaction were separated by electrophoresis on a nondenaturing 6% polyacrylamide gel in low salt TBE buffer containing 4 mM Tris, 44 mM boric acid, and 1 mM EDTA. DNA-protein complexes and unbound DNA probe were visualized in the gel by autoradiography on x-ray film. For the competition experiments, various amounts of unlabeled DNA were added at the beginning of the binding reaction. For gel mobility supershift analysis, the binding reactions were performed as described above, except that the nuclear extracts were incubated for 30 min at 4 °C with variable amounts of specific antisera prior to the addition of the radiolabeled probe. The antisera raised against NF-1, C/EBP, Sp1, Sp2, Sp3, Sp4, c-Jun, c-Fos, JunB, and JunD were purchased from Santa Cruz Biotechnology, Inc.; the NF-YA antibody was purchased from Rockland Inc.

DNA Blotting—Total RNA was isolated from keratinocytes cultured in medium containing 0.05, 0.12, or 1.4 mM Ca2+ for 3–5 min and then maintained either in medium with Ca2+ concentration of 0.05 or 1.4 mM. CAT activities were determined 48 h after transfection and normalized to protein value at A595. CAT activities were measured by the fluorodiox CAT assay using Ecolfluor premixed scintillation fluid and [3H]acetyl coenzyme A (NEN Life Sciences Products) in the linear range of the assay. Results are expressed as the counts/min transferred to the organic solvent phase. Each transfection was done in duplicate, and the experiment was repeated at least three times.

Mapping of the Transcriptional Start Site—To determine the +1 site, we used the rapid amplification of cDNA ends method, as described by Frohman et al. (29). The steps of reverse transcription, tailing, and PCR were performed using the 5'-rapid amplification of cDNA ends system (Life Technologies, Inc.). The gene-specific oligomer was located at +124 bp from the translation initiation site of Dlx3. The final PCR products were cloned and sequenced to determine the extreme 5' mRNA sequence.

Transfection and CAT Assays—Primary mouse keratinocytes were transfected using the Lipofectin reagent (Life Technologies, Inc.). Typically, 1 μg of each CAT construct was used to transfect cells plated and cultured in 6-well plates coated with rat tail collagen (0.1 mg/ml). After 4 h of incubation, cells were treated with 15% glycerol in KSF medium (Life Technologies, Inc.) for 3.5 min and then maintained either in medium with Ca2+ concentration of 0.05 or 1.4 mM. CAT activities were determined 48 h after transfection and normalized to protein value at A595. CAT activities were measured by the fluorodiox CAT assay using Ecolfluor premixed scintillation fluid and [3H]acetyl coenzyme A (NEN Life Sciences Products) in the linear range of the assay. Results are expressed as the counts/min transferred to the organic solvent phase. Each transfection was done in duplicate, and the experiment was repeated at least three times.
Regulation of the Dlx3 Homeobox Gene

FIG. 1. Dlx3 expression is up-regulated during differentiation induced by Ca2+. Northern blot analysis of 2 "ug of total RNA from primary mouse keratinocytes cultured in 0.05, 0.12, and 1.4 mM Ca2+ containing medium for the indicated times (in hours after Ca2+ addition). A 1.1-kilobase pair Dlx3 cDNA fragment was used as a hybridization probe. The same RNA samples were hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe for normalization.

beled with 32P by random priming (Stratagene), and washed at high stringency (0.1 × SSC, 0.1% SDS at 65 °C). The hybridizing bands were visualized by autoradiography on x-ray film and quantitated with a TD932 Macbeth densitometer using linear exposure.

UV Cross-linking Assay—The 5'-end-labeled probe was incubated with primary mouse keratinocyte nuclear extract under standard mobility shift assay conditions in the presence of 500 ng of poly(dI-dC) in a 30-μl binding reaction at room temperature. The reaction mixture was irradiated with an UV transilluminator (254 nm, 2400 microwatts/cm²) for 20 min at 4 °C, and the complex was loaded on a 10% SDS-polyacrylamide gel. The gel was dried, and the proteins directly involved in the DNA interaction were detected by autoradiography.

RESULTS

Induction of Dlx3 Expression by Ca2+ in Mouse Keratinocytes Cultured in Vitro—Northern blot analysis of total RNA from mouse keratinocytes cultured in 0.05 mM Ca2+ medium revealed very low levels of Dlx3 transcript (Fig. 1). However, after 12 h, Dlx3 expression increased 3.5-fold in keratinocytes induced to differentiate by Ca2+ increase (to 0.12 mM). As shown in Fig. 1, the expression increased in a time-dependent manner in 0.12 mM Ca2+ medium, and by 12 h an 8-fold increased expression of Dlx3 was observed in keratinocytes cultured at 1.4 mM Ca2+.

Cloning and Analysis of the Upstream Sequence of the Marine Dlx3 Gene—We cloned two overlapping genomic DNA sequences of the Dlx3 upstream regulatory region (754 bp and 617 bp, see "Materials and Methods"), which combined were cloned as 1416 bp upstream sequence from the translation start site (Fig. 2). The transcription start site was determined using the rapid amplification of cDNA ends procedure (29) and was located 205 bp upstream from the translation start site. Genomic DNA sequence analysis revealed that the upstream region of Dlx3 gene contains canonical CCAAT and TATA boxes. Comparison of the proximal promoters of the Xenopus, human, and mouse Dlx3 genes showed a striking degree of conservation. Particularly the mouse and human upstream regions exhibit about 91% homology in the region located between -104 and +61 (data not shown), suggesting the possibility of conserved mechanisms of regulation for Dlx3 orthologs during evolution.

Deletion Analysis of the Dlx3 Promoter—Comparing the sequence of the −1213 to +160 fragment of the Dlx3 gene with the data base for consensus transcription factor binding sequences, putative sites for AP1, GRE, OCT1, NFκB, and Sp1 were identified (Fig. 2). This information was taken into consideration when serial deletion plasmids were constructed by PCR in order to identify the regulatory elements with a functional role in Dlx3 gene expression. These constructs were transfected into primary mouse keratinocytes, and promoter activity was determined by measuring CAT activity in cells maintained in an undifferentiated state (cultured in 0.05 mM Ca2+) or in cells differentiated in vitro by addition of Ca2+ (1.4 mM Ca2+) (Fig. 3). An increase in activity of all constructs was observed upon differentiation. This increase is coincident with the results obtained for the endogenous gene shown in Fig. 1 by Northern blot analysis. In 0.05 mM Ca2+, the p84CAT construct showed the highest activity, almost 3-fold increased activity when compared with the p162CAT. The p84CAT construct containing the TATA box and putative Sp1-binding site showed a 3-fold decrease in CAT activity when compared with the p84CAT, even though the activity is still higher than that of the full-length promoter (p1213CAT). These results indicated that there are, at least, one negative regulatory and one positive regulatory region in the upstream sequence of Dlx3 gene, which are located between −162/−84 and −84/−34, respectively. The p1213CAT construct showed about 3.5-fold increased transcriptional activity by Ca2+ addition. Similar induction by Ca2+ addition was measured for each deletion construct, and as shown in Fig. 3, the activity of the p34CAT construct is still at least 2-fold higher in 1.4 mM Ca2+ than in 0.05 mM Ca2+.

The Transcription Factor NF-Y Binds to the CCAAT Box and Plays an Important Role in the Dlx3 Promoter—By deletion analysis, we showed that the region between −84 and −34 played a major regulatory role in the activity of the Dlx3 promoter. To examine if a protein bound to this region and define the nucleotides required for the formation of the complex, the 50-bp oligonucleotide (−84 to −34; d oligonucleotide) was used as a probe in gel shift analysis, and three overlapping oligomers (d1–d3) were used as competitors (Fig. 4A). One major complex was found, and by competition analysis, binding was located to the proximal part of the region (between −84 and −59) (Fig. 4B). Better resolution of the specific nucleotides required for formation of the complex was obtained using oligonucleotides with specific 3-bp mutations (Fig. 4C). Competi-
tion gel shift analysis with wild-type (d1) and mutant oligonucleotides (M1–M6) showed that competitors M1, M2, and M3 bound to the specific complex (Fig. 4D). In contrast, oligonucleotides M4, M5, and M6 (which has the combined mutation of M4 and M5) failed to compete. These results indicated that the complex was due to binding in the CCAAT region (Fig. 5B). To determine the nature of the binding, oligonucleotides that have been reported to bind selectively the CCAAT box binding proteins were used as competitors (Fig. 5A). The CCAAT box binding factors include NF-Y (also known as CBF), the C/EBP family, and NF-1 (35). As shown in Fig. 5E, the albumin C element, which is known to bind NF-Y (36), fully competed the formation of the complex (Fig. 5B, left panel, 3rd lane). In contrast, the consensus NF-1 and C/EBP oligonucleotides did not compete.
NF-Y is a heterotrimeric transcription factor composed of three subunits, A, B, and C (37–43). The subunits A and C together form a heterodimer, which then interacts with the subunit B to form the heterotrimeric NF-Y protein. This heterotrimeric NF-Y then binds to DNA to form an NF-Y-DNA complex. To examine whether the complex consisted of all three subunits, we checked the molecular weight of the complex using the UV cross-linking assay (Fig. 5D). The complex (indicated by arrow) which competed with self-oligonucleotide (d1) had an estimated molecular mass of ~100 kDa. This molecular mass coincides with the approximate expected combined molecular mass of subunits NF-YA, -B, and -C (35, 25, and ~40 kDa, respectively) (44). This result strongly supported that NF-Y was binding to the CCAAT box motif of Dlx3 promoter in its heterotrimeric form.

The function of the Dlx3 promoter CCAAT box was assessed by mutational analysis. Primary mouse keratinocytes were transfected with the wild-type p84CAT construct, the mutant p84mCAT construct, and the p34CAT construct (Fig. 6A). The CAT activity conferred by each construct was assayed in transiently transfected cells grown in 0.05 and 1.4 mM Ca²⁺ (Fig. 6B). The CAT activity of the mutant construct was reduced to 40 and 25% as compared with the wild-type p84CAT construct in the undifferentiated and differentiated cells, respectively. These results underscore the functional importance of the CCAAT box for the activity of the Dlx3 promoter.

Functional Sp1-binding Site in the Dlx3 Promoter—In addition to CCAAT box sequence, analysis of the proximal promoter region revealed a putative recognition site for Sp1 transcription factor, which was located between the TATA box and transcription start site (−13 to −3). To examine this putative Sp1-
Defining the Region of the Dlx3 Promoter Responsible for Ca$^{2+}$ Inducibility—Despite the finding that the binding sites for NF-Y and Sp1 play an important role in the transcriptional regulation of the Dlx3 gene, mutation of these sequences did not affect the activation of Dlx3 by Ca$^{2+}$ (Fig. 6 and 8). Serial 3’-deletion constructs were made on the p34CAT construct, which contains the 160 bp of untranslated region (Fig. 9A). The constructs were made in an attempt to delineate the region responsible for the Ca$^{2+}$ inducibility. Each construct was transfected into primary mouse keratinocytes, and CAT activity was measured in 0.05 and 1.4 mm Ca$^{2+}$ conditions (Fig. 9A). The p60(+)-CAT deletion retained the capability of induction by Ca$^{2+}$, but the p10(+)CAT deletion showed no induction of CAT activity with Ca$^{2+}$ increase (Fig. 9A). Similar 3’-deletions were made on the p1213CAT construct. These constructs were transfected into primary mouse keratinocytes (Fig. 9B), and the CAT activity indicated that the p1213(+160CAT as well as the p1213(+30CAT were induced 2.5-fold, whereas the p1213(+30CAT was induced only 1.5-fold. In contrast, the p1213(+10CAT deletion was not induced with Ca$^{2+}$. Our findings indicated that the region located between +10 and +60 was dispensable for induction by Ca$^{2+}$ of the Dlx3 promoter.

Characterization of the Ca$^{2+}$-responsive Region of Dlx3 Promoter—Based on the results of the serial deletion analysis of the Dlx3 promoter (Fig. 9), the region between +30 and +60 has the element(s) that mediate the Ca$^{2+}$ induction. A mutational analysis was performed to determine the specific nucleotides that conform the responsive element. Serial and double 4-bp mutations were made in the region between +30 and +60 in the context of the p1213(+160CAT construct. These constructs were transfected into primary mouse keratinocytes, cultured for 48 h in 0.05 or 1.4 mm Ca$^{2+}$ concentrations, and the CAT activity was measured. As shown in the Fig. 10A, the M1CAT, M2CAT, M5CAT, and M7CAT showed reduced promoter activity at 0.05 and 1.4 mm Ca$^{2+}$, but the fold activation by Ca$^{2+}$ did not change significantly when compared with the p1213(+160CAT. In contrast, the M4CAT and M6CAT showed increased promoter activity in 0.05 and 1.4 mm Ca$^{2+}$ and slightly increased fold activation by Ca$^{2+}$. These results suggest that these sites mainly affect the basal promoter activity of Dlx3. Interestingly, the M3CAT construct showed dramatic reduction in the fold activation by Ca$^{2+}$, similar to that of p1213(+30CAT. These results indicated that the nucleotides (+42-CGAC-+45) are crucial for the activation by Ca$^{2+}$. Double mutant constructs (M34CAT, M36CAT, and M46CAT, Fig. 10A) were made and assayed for CAT activity. The double mutant constructs containing the M3 site (M34CAT and M36CAT) showed reduced fold activation by Ca$^{2+}$, but the M46CAT mutation had no effect. These results again delineated the sequence (+42-CGAC-+45) as primarily responsible for the Ca$^{2+}$ induction of Dlx3 promoter.

We were interested in determining by gel shift analysis if there were nuclear proteins that bound the +30/+60 region (CA30). The CA30 oligonucleotide was used as a probe in gel shift mobility assays with nuclear extracts from mouse primary keratinocytes. One major complex I and two weak bands II and III were identified (Fig. 10B). These complexes were competed with 100-fold excess of unlabeled cold CA30 oligomer. To examine the relationship between the sequences affecting the Dlx3 promoter activity and these complexes, the oligomers containing each mutant site were used as competitors. The M3
oligomer competed the complexes II and III, but did not completely compete the major complex I. The M4, M6, and M34 oligomers showed similar results for the complex I but did not compete the complexes II and III. In contrast, none of the complexes were competed by the M36 oligomer that contains mutations in the M3 and M6 sites. These results indicated that the M3 and M6 sites are important in the binding of the major complex I and that the M4 site is partially involved in the formation of this complex.

The region between 130 and 160 contains an element (146-TGACCGA-152) with sequence homology to a consensus AP1-binding site. It has been suggested that the AP1 transcription factor is involved in the Ca2+ inducibility of epidermal differentiation-specific markers (45). To determine the possible participation of AP1 in the protein-DNA complex formation with CA30, the AP1 consensus oligonucleotide and antibodies against AP1 family members were used in competition and supershift assays. As shown in Fig. 10C, the complexes formed by CA30 were not competed by AP1 consensus oligonucleotide, and the supershift assays corroborated that AP1 family members (c-Jun, c-Fos, JunB, and JunD) do not bind to this region. Therefore, the sequence (+42-CGAC-+45) present in the CA30 region is responsible for the transcriptional up-regulation of Dlx3 promoter by Ca2+, through the binding of nuclear factors that are not members of the AP1 family of transcription factors.

FIG. 8. Mutational analysis of the Sp1-binding site in the Dlx3 promoter. The diagram (left) illustrates the p84CAT, p34CAT, p84spmCAT, and p34spmCAT deletion and mutant constructs. The sequence of the mutation of the Sp1-binding site is the same shown in Fig. 7A. Primary mouse keratinocytes were transfected with p84CAT, p34CAT, p84spmCAT, or p34spmCAT, incubated with growth medium containing 0.05 or 1.4 mM Ca2+, and harvested after 48 h. The CAT activity was normalized to the protein concentration measured by the Bradford assay. The bars represent the average normalized CAT activity of duplicate plates for each construct (right panel). All experiments in this figure were repeated three times with similar results.

FIG. 9. Deletion analysis and function of sequence residing downstream of the transcription initiation site. A, p34CAT constructs with decreasing downstream promoter deletions were transfected into primary mouse keratinocytes and incubated with growth medium containing either 0.05 or 1.4 mM Ca2+. CAT activity was assayed 48 h after transfection. B, p1213CAT constructs with decreasing downstream promoter deletions were transfected into keratinocytes and assayed as described above. The bars represent the average normalized CAT activity of duplicate plates for each construct. All experiments in the figure were repeated in triplicate with similar results.

DISCUSSION

The cascade of events that leads to terminal differentiation can be triggered in primary mouse keratinocytes by the elevation of extracellular Ca2+. The expression of the Dlx3 homeobox gene is restricted to the suprabasal layer of the epidermis (18), and this expression is dramatically increased in primary mouse keratinocytes induced to differentiate by Ca2+ in vitro. Evidence obtained in transgenic mice suggests the role of Dlx3 as a positive transcriptional activator of differentiation-specific epidermal structural genes. In an attempt to decipher the different stages of the pathway that culminate in terminal differentiation, we have cloned and characterized the upstream promoter sequence of the murine Dlx3 gene, and we determined the regulatory elements necessary for expression of the gene in keratinocytes. By sequence comparison we have found that the region close to the transcription start site of the mouse Dlx3 gene (−110 to +61) has striking homology with that of the Xenopus and human dlx3 genes. In transgenic mice, 470 bp of the Xenopus Dlx3 promoter conferred an expression pattern to the β-galactosidase reporter that was indistinguishable from that of the endogenous gene, including the Ca2+ response (46). Thus, it could be hypothesized that the cis- and trans-regulatory elements important in the regulation of the Dlx3 orthologs have been conserved through evolution and are contained within the proximal promoter region.

To understand the mechanism(s) that control the regulation
FIG. 10. Characterization of Ca\(^{2+}\)-responsible region of Dlx3 promoter. \(\text{A,} \) the region between +30 and +60 of Dlx3 promoter was mutated with serial or double 4-bp modified bases by the PCR method in the p1213(+60)CAT. Each mutated construct, p1213(+60)CAT, p1213(+30)CAT, and p1213(+10)CAT, was transfected into primary mouse keratinocytes. Underlined lowercase letters indicate the modified nucleotides. CAT assay was performed the same as in Fig. 3. The bars represent the average normalized CAT activity of duplicate plates for each construct. All experiments in the figure were repeated in triplicate with similar results. \(\text{B,} \) gel shift assay was performed using the oligonucleotide corresponding to the region between +30 and +60 (CA30) as a probe and 3 \(\mu\)g of nuclear extract of primary mouse keratinocytes. Incubations were carried out in the presence or absence of a 100-fold excess of unlabeled wild-type and mutant oligonucleotides. Arrows indicate the protein-DNA complexes, and roman numbers indicate each individual complex (I–III). \(\text{C,} \) supershift analysis was carried out using the antibodies against the AP1 family. Nuclear extracts were preincubated with 2 \(\mu\)g of each antibody as indicated, followed by incubation with labeled CA30 probe. The 3rd lane corresponds to the gel shift analysis performed in the presence of 100-fold excess unlabeled AP1 consensus oligonucleotide. All experiments in this figure were repeated at least three times with similar results.
and expression of the Dlx3 promoter during epidermal differentiation, our studies examined the expression of Dlx3 promoter/CAAT constructs in primary mouse keratinocytes to identify putative regulatory elements that are required for Dlx3 expression. The data presented here demonstrate the existence of one positive regulatory region in the Dlx3 promoter located between −84 and −34. We determined that the CAAT box in this region exerts a significant positive influence on the Dlx3 promoter activity in undifferentiated and differentiated keratinocytes. Competition gel shift analysis and supershift analysis further demonstrated that the transcription factor NF-Y is the protein that binds specifically to the CAAT box motif. Mutation of this motif dramatically reduced transcription of the Dlx3 promoter. Several genes also contain canonical sites for CAAT-binding proteins that have been described to be important in early functions of preinitiation complex formation (36). CAAT boxes are typically located within 100 bp of the transcription start sites, and CAAT box binding factors include NF-Y (also known as CBP), C/EBP family members, and NF-1 (35). NF-Y was originally identified as a ubiquitously expressed protein that binds to the Y box motif, defined as an inverted CAAT box motif in all major histocompatibility complex class II genes (47). NF-Y is a heterotrimeric transcription factor composed of three subunits (37–43). Formation of a complex between the A and C subunits is required to bind the B subunit, and together, the heterotrimeric complex binds DNA (48). The activation domains of both B and C subunits, which are rich in glutamine and hydrophobic residues, share protein sequence homology with each other and with the glutamine-rich activation domain of the transcription factor Sp1 (48).

NF-Y has been demonstrated to act as both a positive (49–51) and a negative (52) regulator of transcription. Several studies suggest that NF-Y acts by stabilizing the binding of additional factors to adjacent regulatory elements, such as RXF in the major histocompatibility complex class II promoter (53, 54). NF-Y also interacts with transcription factors binding upstream elements and basal transcription machinery, such as HNF4 and TAF100 (42, 55). Recently it was shown that the interaction of NF-Y with cAMP-response element-binding protein may be responsible for the gene-specific transcriptional activity of the insulin cAMP-response element (56), and similar results have been reported for the albumin promoter (36). Since the expression of Dlx3 gene is epithelial specific and the activity of the Dlx3 promoter is low in the fibroblast cell line NIH 3T3 (data not shown), NF-Y and some interacting regulatory factors may be involved in conferring tissue- and differentiation-specific expression to the Dlx3 gene.

In this study, we also demonstrate that the Sp1-binding site located between −21 and +2 positively regulates, and contributes to, the basal activity of the Dlx3 promoter in undifferentiated and differentiated keratinocytes. The Sp1 consensus motif is highly GC-rich; therefore, certain Sp1-binding sites can overlap with other GC-rich consensus transcription factor binding motifs such as that for Ap-2. The ratio of Sp1/Ap2 might be determinant in the differentiation-specific gene expression (57). Many epidermal specific gene promoters contain consensus sites for ubiquitously expressed regulators such as Sp1 family members. The Sp1 family consists of four members (Sp1–Sp4; see Refs. 58–60). Sp1 is involved with tissue/cell type-specific expression of certain genes in epithelial cells. In most of these cases, Sp1 alone does not affect tissue specificity but requires cooperation with other transcription factors, such as Ets in the promoter of human transfglutaminase 3 (61), Ap-2 in the K3 keratin gene (57), Egr-1 in the tissue factor gene (62), and NF-1 in the mouse vas deferens protein promoter (63).

From the family of Sp1-related transcription factors, studies have shown that Sp3 acts as an antagonist to Sp1. The human transcriptional enhancer II gene is controlled by the relative ratios of Sp1 and Sp3 (64), and activation of the human papilloma virus type 16 promoter correlates with the ratios of Sp1/Sp3 during epithelial differentiation (65). We have shown that the Sp1 consensus motif on the Dlx3 promoter can bind specifically Sp1 and Sp3, providing the possibility of antagonistic effects on transcription depending on the specific levels of each of these factors throughout the terminal differentiation process.

An essential aspect of the transcriptional regulation of the Dlx3 promoter is the elucidation of the mechanism for inducing its expression by increases in external Ca2+ during differentiation. Until now, the prevailing evidence points to the involvement of PKC isozymes in the induction of keratinocyte differentiation markers by Ca2+, although the exact mechanism is unclear. In this study we show that the region located between +10 and +60 is important for Ca2+ inducibility of the Dlx3 gene. By mutational and gel shift analysis of the +30/+60 sequence, we found that the crucial element responsible for Ca2+ inducibility is located between +42 and +45 (CGAC) and that nuclear factor(s) are involved in the up-regulation of Dlx3 expression by Ca2+. It has been reported that the AP1 and Ets transcription factors are involved in the regulation of the human SPRRIA keratinocyte terminal differentiation marker (45). However, in the case of the Dlx3 gene, the AP1 transcription factor does not bind to the +42/+45 or +46/+52 sequences, which strongly supports that it is not the determinant for the Ca2+ inducibility of Dlx3. Future work will determine the specific nature of the nuclear factor(s) involved in the binding to this regulatory element. The identification of a Ca2+ response element in the Dlx3 gene is the first link between the extracellular signal and the transcriptional control of a regulatory gene involved in keratinocyte differentiation, and represents an important step in the elucidation of the molecular mechanisms underlying this developmental program.

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