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Down-Regulation of Human Sialyltransferase Gene Expression during in Vitro Human Keratinocyte Cell Line Differentiation

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Sialic acids play important roles in biological processes, such as cell-cell communication and cell-matrix interaction. Histochemical analysis using PNA and LFA lectin has shown that the expression of α2,3-sialic acid linked to Galβ1,3GalNAc is high in basal cells and decreases following further keratinocyte differentiation. In the present study, we used an in vitro keratinocyte cell line differentiation model to study expression of α2,3-sialic acid linked to Gal β1,3 GalNAc. Treatment of the human papillomavirus type 16-immortalized human keratinocyte (PHK16) cell line with high concentrations (1.0 mM) of Ca^{2+} resulted in PHK16 cell differentiation and redistribution of PNA binding glycoproteins. The synthesis of α2,3-sialic acid linked to Galβ1,3GalNAc is mediated by three β-galactoside α2,3-sialytransferases, which are the gene products of hST3O, hST3O/N and hST3 Gal II. Ca^{2+} treatment of PHK16 cells decreased the mRNA expression of hST3O/N, whereas the mRNA of hST3O and hST3Gal II was not detected by Northern blot analysis, suggesting that the hST3O/N gene is responsible for sialic acid down regulation during keratinocyte differentiation. In order to examine transcriptional regulation of the hST3O/N gene, we first determined the transcriptional starting sites of the hST3O/N gene in PHK 16 using 5′-RACE analysis. Two kinds of type B isoforms, types B3 and BX, were identified. Type BX is a novel isoform related to the type B form, but which differs upstream of the B3 exon. The results of Northern blot analysis using a type BX-specific probe suggest that the B3 promoter may be regulated by Ca^{2+}. Using a luciferase assay, we identified a functional DNA portion within hST3O/N genomic DNA that confers negative transcriptional regulation on the hST3O/N B3 promoter during Ca^{2+} stimulated human keratinocyte differentiation. This element contains some putative transcriptional factor binding sequence motifs such as AP2.

Sialic acids are key determinants for biological processes, such as cell-cell interaction and cell differentiation (1,2). The expression of oligosaccharide structures occurs in differentiation dependent patterns (3,4). Peanut agglutinin (PNA; carbohydrate specificity : Galβ1,-3GalNAc) has been proven to be remarkably useful as a differentiation marker, both in vivo and in vitro cultured keratinocytes. PNA binds more strongly to suprabasal than basal keratinocyte (5–8) and also shows specificity for terminally differentiating keratinocytes in vitro when stratification is induced by increasing the extracellular concentration of calcium ions (7,9). However, a sialic acid specific lectin, *Limax flavus* agglutinin (LFA) shows strong binding affinity to basal keratinocyte (10,11). These results demonstrate that the expression of α2,3-sialic acids linked to Galβ1,3-GalNAc is high in basal cells and decreases following further keratinocyte differentiation.

The synthesis of α2,3-linked sialic acid to Galβ1,3-NAc is mediated by the three β-galactoside α2,3-sialyltransferase (EC 2.4.99.4) gene products of hST3O (12), hST3O/N (13,14) and hST3 Gal II (15,16). The structure and chromosomal location of these genes have been determined (17,18). The mRNAs of hST3O/N in human placenta consist of five isoforms, namely type A1, A2, B1, B2, and B3 (17). These transcripts are produced by a combination of alternative splicing and promoter utilization, suggesting that the transcriptional regulation of this gene depends on the use of alternative promoters. The expression of sialyloligosacchar-

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ides is controlled in part by sialyltransferase. To date, five human α2,3-or α2,6-sialyltransferase genes have been cloned (12–14, 18–20). However, transcriptional regulation of these genes is not well understood, and only the transcriptional mechanism of the α2,6-sialyltransferase gene has been reported, resulting in the proposal that tissue-specific expression of this gene depends on the use of alternative promoters (21,22).

In keratinocytes, as in other cell types, the expression of most genes is regulated at the transcriptional level by a class of proteins called transcription factors. Several transcription factors which are involved in keratinocyte-specific gene expression have been characterized, such as activator protein 1 (AP1), AP2, Sp1, and ESE-1 (for epithelium-specific Ets) (23–25). Expression of the keratin K5 and K14 genes is high in undifferentiated basal cells and decreases during further differentiation (26), similar to the case of sialic acids linked to Galβ1,3GalNAc. AP2 is likely to contribute to keratinocyte-specific expression of K5 (27) and K14 (28).

In present report, we determined that differentiation elicits negative regulation of hST3O/N at the mRNA level in a human keratinocyte cell line. Analysis of transcriptional regulation of the hST3O/N gene upon differentiation of this cell line revealed a functional DNA portion within the hST3O/N genomic DNA that confers negative transcriptional regulation on the hST3O/N B3 promoter.

METHODS

Cell culture. A HPV 16-immortalized human keratinocyte (PHK16) cell line has been established in our laboratory and is maintained in complete MCDB 152 medium containing epidermal growth factor (EGF, 10 ng/ml), insulin (5 μg/ml), transferrin (10 μg/ml), hydrocortisone (0.2 μM) and 0.5% chelated fetal calf serum as described (29,30). Normal human keratinocyte (NHK) was purchased from Kyokuto Seiyaku Co., Ltd (Japan), and maintained in K100 medium.

Western blot analysis using PNA lectin. Western blot analysis was performed according to Ochiai et al. (31). Cell surface proteins were extracted from 3×10^4 cells using lysis buffer (PBS containing 1 % Triton X-100) separated by 7.5 % SDS-PAGE, and electroblotted to nitrocellulose membrane. The nitrocellulose sheet was treated with 2.5 μg/ml of PNA-peroxidase (Honen Oil Company, Japan), and washed with washing buffer (10 mM Tris-HCl, pH 7.5 containing 0.05 % Tween-20 and 0.15 M NaCl) three times, and then positive bands were visualized using Konica immune-stain (Konica, Japan).

Northern blot analysis. Fragments generated by PCR amplification of cDNA (for hST3O, amplification of HT-29 cDNA, for hST3O/N amplification of COLO201 cDNA, for hST3Gal II amplification of Hu-H7 cDNA) using specific primers were used as the probes in Northern blot analysis. The specific primers were 5′-CACCATCTGGTGTACCCTGAG-3′ and 5′-TCATCTCCCCTTGAAGATCCG-3′ for hST3O, 5′-ATGCGTCTCTTCTACCCTGAATCT-3′ and 5′-TCAGAAGGACGTGAGGTTCTTGAT-3′ for hST3O/N, and 5′-ACCATGAAGTGCTCCCTGCGGGTGTGGTTC-3′ and 5′-GGCTCAGTTGCCCCGGTAGACTTCGATCTT-3′ for hST3Gal II. The amplification products were cloned into a TA cloning vector (Invitrogen, CA) and were verified by sequence analysis. The nucleotide sequence of the BX-specific probe was 5′-ACGGGGTGGAGCGGGGCGGGCGACAGCGGTTCCGCGCAGCTCCGAT-3′.

Twenty μg of total RNA, extracted by the RNA zol B (Biotecx Laboratories,TX) method, was fractionated on a formaldehyde agarose gel, and capillarily transferred to a nitrocellulose membrane (Schleicher and Schuell, Germany). Hybridization was performed as previously described (32). The sialyltransferase cDNA fragments were labeled with ^{32}P using the nick translation system (GIBCO

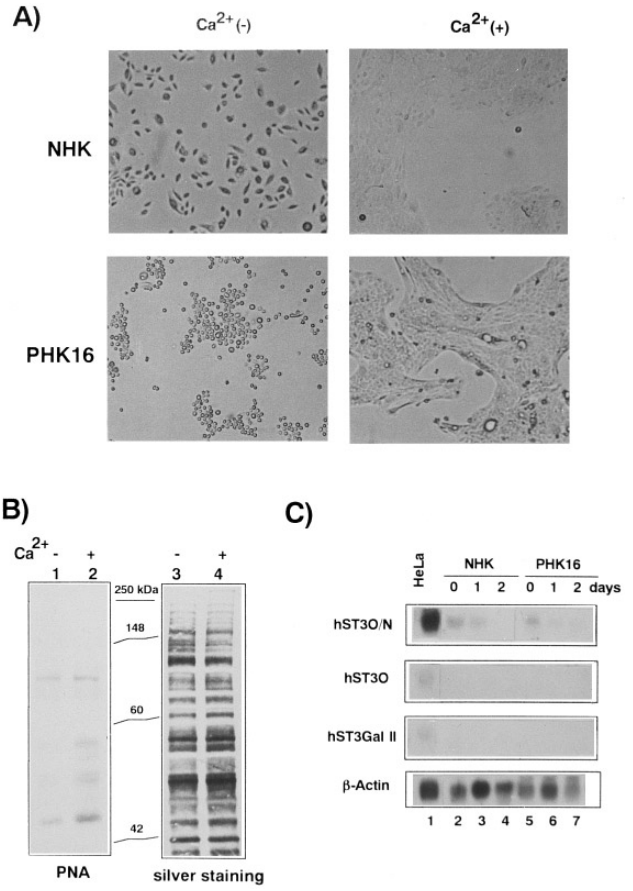

FIG. 1. The hST3O/N gene was down-regulated during differentiation of PHK16 and NHK cells. (A) Morphological changes of NHK and PHK16 cells in the presence of high concentrations of Ca^{2+}. Left panels show the morphology of NHK and PHK 16 cells grown as a monolayer in low Ca^{2+} (0.03 mM) medium. The right panels show the island formation induced by high concentrations of Ca^{2+} (1.0mM). Original magnification, × 13.4. (B) Effect of Ca^{2+} on PNA binding protein expression in PHK16. The PNA binding proteins were detected by lectin blot analysis (left panel). The right panel shows the total protein using silver staining as the control. The membrane proteins were extracted from PHK16 cells grown for 2 days in either low Ca^{2+} (0.03 mM) medium (lane 1 and 3) or high Ca^{2+} (1.0 mM) medium (lane 2 and 4). (C) The mRNA expression of hST3O/N, hST3O and hST3Gal II genes by treatment with a high concentration of Ca^{2+}. Northern blotting of mRNA extracted from NHK (lanes 2–4) and PHK16 cells (lanes 5–7) grown in a low Ca^{2+} (0.03 mM) medium (lanes 2 and 5) or high Ca^{2+} (1.0 mM) medium for 1 day (lanes 3 and 6) or 2 days (lanes 4 and 7). The mRNA from HeLa cells was used as the positive control (lane 1). The blot was probed with ^{32}P-labeled hST3O/N (upper panel), hST3O (second panel), hST3Gal II (third panel), or β-actin (bottom panel) cDNA.

BRL, MD). Oligonucleotide probe was labeled with [γ-^{32}P]ATP using T4 kinase. The hybridized blots were washed twice at room temperature in 2 × SSC, 0.1 % SDS for 10 min, then twice at 42 °C in 0.2 × SSC, 0.1 % SDS for 30 min, and exposed to x-ray film for several days at −80°C.

PCR Amplification of the 5′-cDNA end (RACE). Amplification of the 5′ end of hST3O/N cDNA was performed according to the manufacturer's instruction (5′-full RACE core set, Takara, Japan) (33). First-strand cDNA was synthesized from 3 μg of total RNA using the gene specific primer, 5′-pCAGCTCGTAAGCAGA-3′. After digestion of the template mRNA with RNase H at 30 °C for 1 hr, cDNA was precipitated with ethanol and ligated using T4 RNA ligase at 16 °C for 16 hr. A 1 in 10 dilution of the ligation mixture was used as the template for the first PCR amplification using 5′-CTCCTTCTTCTCTGGGATGGGAAA-3′ as the sense primer and 5′-CTCCAGGGTGAGGCAGAGAGCAAGGCC-3′ as the anti-sense primer. Twenty five cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min were performed. The resulting PCR products were diluted 100-fold with sterile water, and amplified under the same conditions using 5′-CCACACCATGACGACCAGGACCAGAGC-3′ as the sense primer and 5′-ATCTTCCTGCGGCTTGAGGATTATTTC-3′ as the anti-sense primer. The PCR products were subcloned into the pCR2.1 vector using the Original TA cloning kit (Invitrogen, CA), and then sequenced using a sequencing kit (USB,OH).

Construction of plasmids for luciferase assay. PCR of the hST3O/N promoter from PHK16 genomic DNA was accomplished using primers that contained a previously reported human hST3O/N promoter sequence (17). The oligonucleotides primers designed and used were (pGL-1402B3: 5′-GGCTCGAGGCCATACTCAGGCTGACCAAGG-3′: pGL-1286B3: 5′-GGCTCGAGTGGATGGAAGAAACTGGCACT-3′, pGL-1050B3: 5′-GGCTCGAGCCAGCTCCCTGTGCTCCCCAT-3′, pGL-920B3: 5′-GGCTCGAGATGTCATAGCCCCACCCTC-3′, pGL-720: 5′-GGCTCGAGAGCTGTCTGCCTCCTGGAGC-5′, pGL-520: 5′-GGCTCGAGCTGGATGAGAAAACTCACC-3′, pGL-420: 5′-GGCTCGAGTCTAGGCAGGAGAGTTTGTG-3′ and 5′-CCAAGCTTATCCCGGCTGCCTCGGGCCA-5′) and (pGLPRO520-420 and pGLPRO420-520: 5′-GGCTCGAGCTGGATGAGAAAACTCACC-3′, and 5′-CCGAGCTCACTGGAGCAGGACACACC-3′). Restriction sites that were incorporated into the primers are underlined. Twenty five cycles of PCR amplification consisting of denaturation at 98 °C for 20 sec, and annealing and extension at 68 °C for 1 min was carried out in a Parkin Elmer / Cetus thermal cycler. A single band was obtained by agarose gel electrophoretic analysis. The PCR products were digested using the *Xho* I and *Hind* II restriction enzymes and cloned into the *Xho* I and *Hind* III sites of the pGL3-Basic Vector or into the *Xho* I site of the pGLPRO vector (Promega, WI). The identification of the amplification products was verified by sequence analysis.

Luciferase assay. Transient transfection of PHK16 cells was performed using TransIT polyamine transfection reagents (PanVera Corporation, WI). Cells were plated at a density of approximately 1-3 × 10^5 cells per 35 mm dish, and then transfected with 1 μg of the pGL constructs and 0.1 μg of pRL-CMV (Promega, WI) as an internal control for variations in transfection efficiency. The pRL-CMV vector contains the CMV promoter located downstream of the *Renilla* luciferase gene. Twenty-four hours after transfection under the low or high Ca^{2+} conditions, cells were harvested and cell lysates prepared. The *Firefly* and *Renilla* luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, WI).

RESULTS AND DISCUSSION

Expression of PNA-binding glycoproteins increases during calcium-induced keratinocyte cell line differenti-

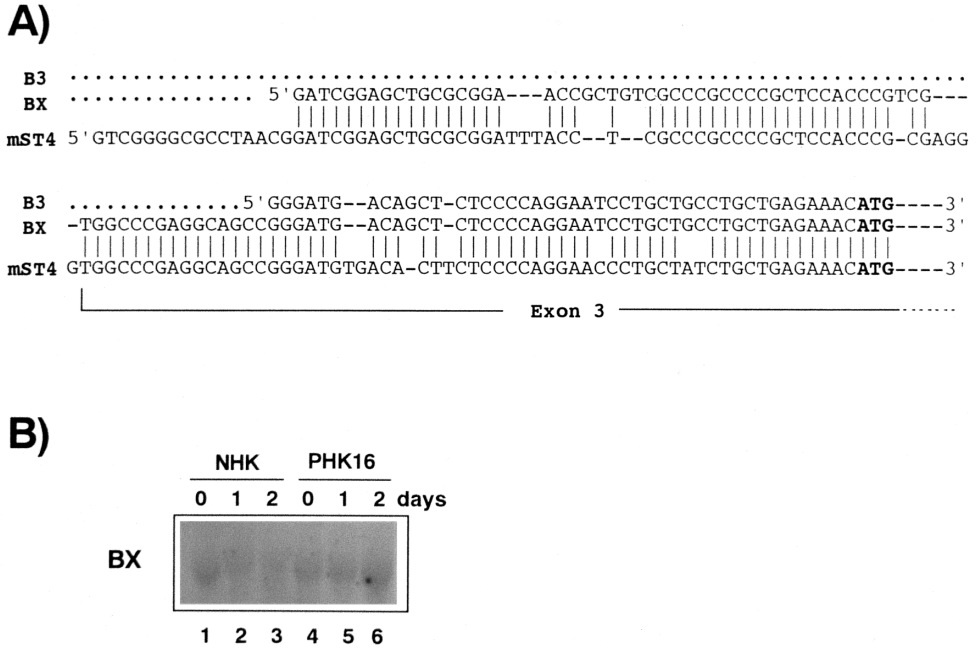

FIG. 2. The isoform responsible for differentiation is type B3 in PHK16 and NHK cells. (A) Comparison of the nucleotide sequences of the 5′-region of type B3, type BX and mouse α2,3-sialyltransferase cDNA (mST4). The nucleotide sequence of BX has been submitted to the GeneBank/EMBL Data Bank with Accession Number AB009393. Initiation codons are represented by boldface letters. (B) Effect of Ca^{2+} on the type BX mRNA isoform in PHK16. The blot used was the same one as in figure 1C and was probed with ^{32}P-labeled BX-specific probe. Northern blotting of mRNA extracted from NHK (lanes 2–4) and PHK16 cells (lanes 5–7) growing in a low Ca^{2+} (0.03 mM) medium (lanes 2 and 5) or high Ca^{2+} (1.0 mM) medium for 1 day (lanes 3 and 6) or 2 days (lanes 4 and 7).

ation. The molecular mechanism by which α2,3 sialic acid linked to Gal β1,3 GalNAc is expressed exclusively in basal cells and disappears as a result of down-regulation during keratinocyte differentiation was examined in the present study using an in vitro keratinocyte cell line differentiation model. The PHK16 cell line used was established in our laboratory and harbors full-length HPV-16 genomes (32,34). This PHK16 cell line stably proliferates in the absence of both high concentrations of Ca^{2+} and serum (32). In the present study, treatment of NHK and PHK16 cells with high concentrations (1.0 mM) of Ca^{2+}, resulted in the induction of cell-to-cell adhesion as well as changes in NHK and PHK 16 morphology to a differentiation-like phenotype (Fig. 1A). To evaluate the PNA binding protein expression during in vitro keratinocyte cell line differentiation, we performed Western blot analysis using PNA lectin with cell surface proteins derived from undifferentiated PHK16 and from PHK16 at 48 hr after induction with calcium. As shown Fig. 1B, the expression of PNA binding glycoproteins increased upon Ca^{2+} treatment, compared with non-treated cells, indicating that α2,3 sialic acid linked to Galβ1,3GalNAc is down regulated during Ca^{2+} induced differentiation of PHK16 cell line. Thus this in vitro system is suitable for investigation of the molecular mechanisms of sialic acid expression.

Down-regulation of hST3O/N mRNA expression during in vitro differentiation of human keratinocyte cell lines. The synthesis of α2,3 sialic acid linked to Galβ-1,3GalNAc is mediated by the three β-galactoside α2,3-sialyltransferase gene products of the hST3O, hST3O/N and hST3 Gal II genes. In order to determine the sialyltransferase gene responsible for differentiation, Northern blots containing mRNAs from PHK16 and NHK cells at different times after induction with high concentrations of Ca^{2+} were probed with the hST3O/N, hST3O, and hST3Gal II cDNA. Expression of hST3O/N was detected in undifferentiated NHK and PHK16

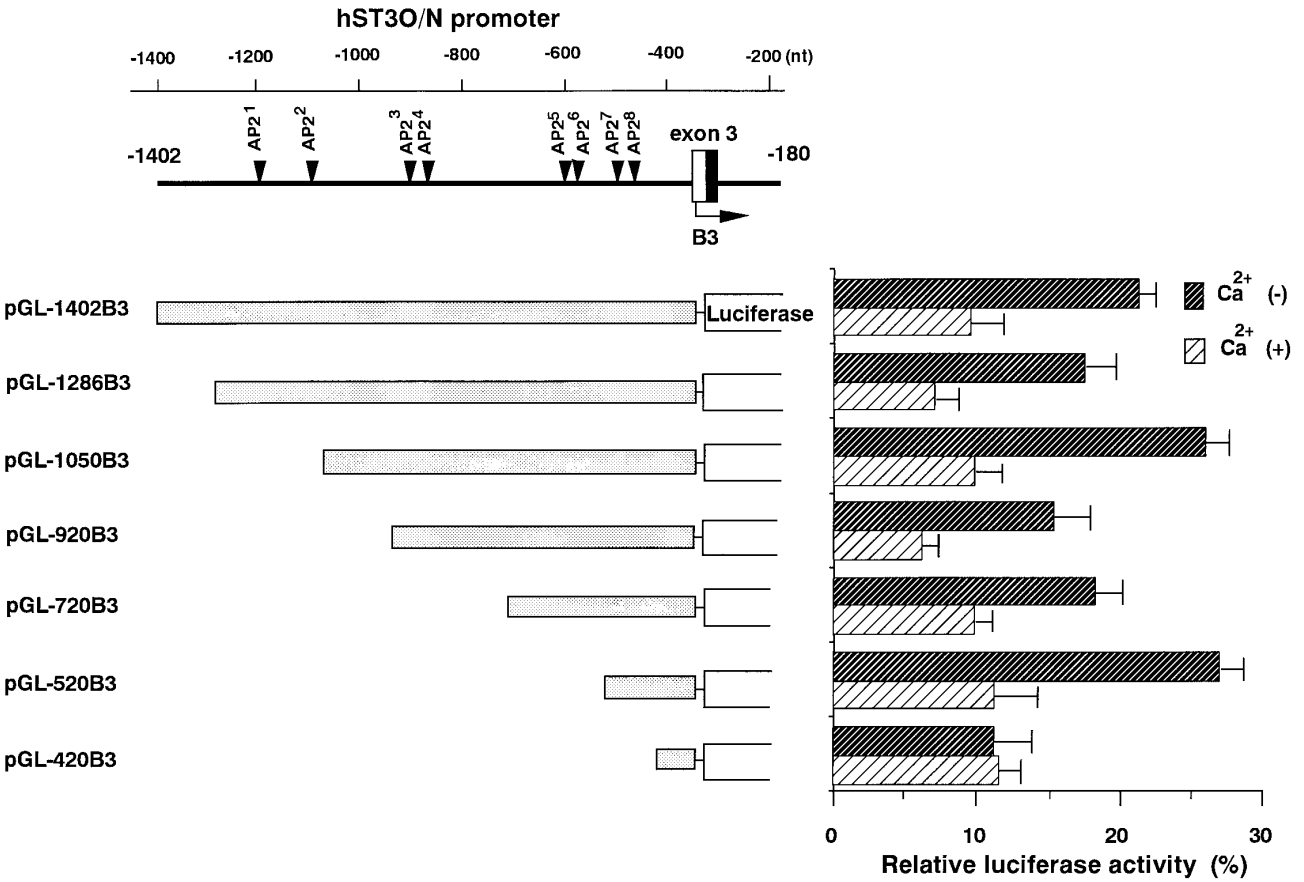

FIG. 3. Deletion analysis of hST3O/N B3 promoter. The structure of the hST3O/N gene is shown above the luciferase constructs. The arrows indicate the AP2-like sequence. The relative luciferase activity of each of these constructs is indicated on the right. Each Firefly luciferase construct was cotransfected into PHK16 cells with the *Renilla* luciferase expression vector (pRL-SV40) as the internal control. Relative luciferase activities were normalized to the luciferase activity of the pGL3-Control, which contains the SV40 promoter-enhancer sequences upstream of the luciferase gene. Data express the mean ± standard deviation (n=3).

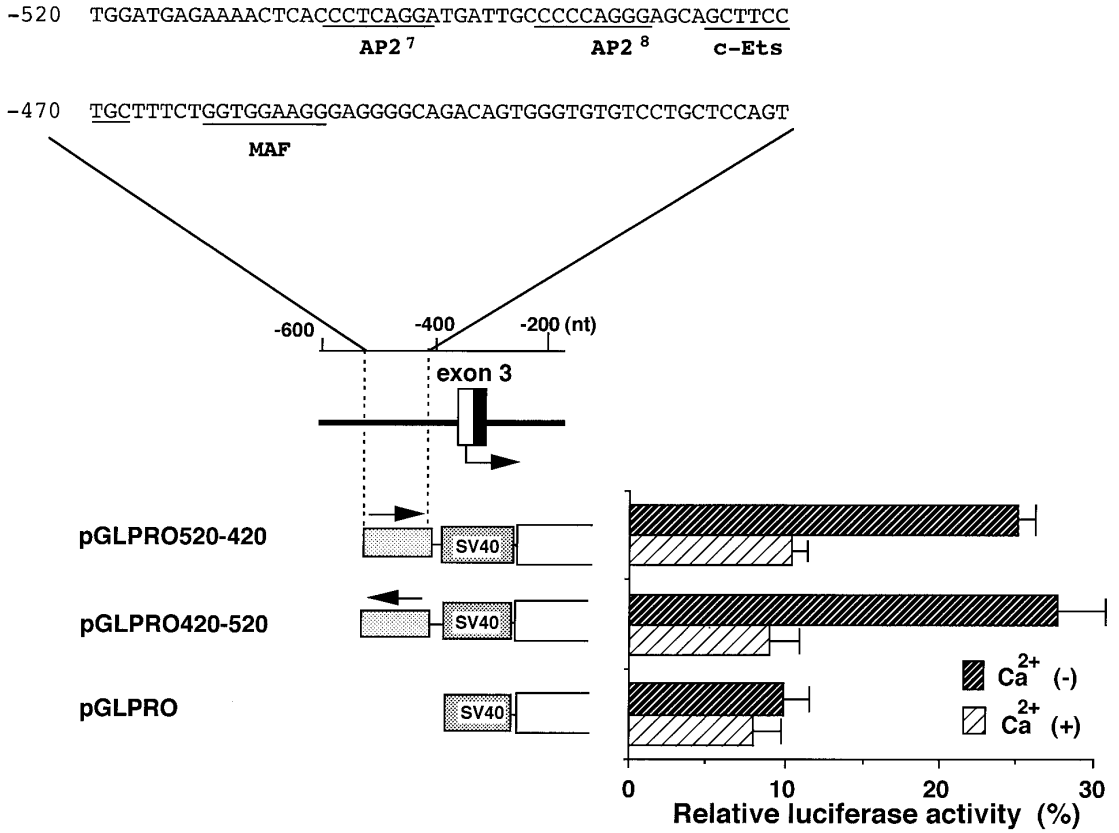

FIG. 4. Differentiation responsive element of hST30/N promoter. The orientation of the nt 520−420 fragment is indicated by → or ←. Each Firefly luciferase construct was cotransfected into PHK16 cells with the *Renilla* luciferase expression vector (pRL-SV40) as the internal control. Relative luciferase activities were normalized to the luciferase activity of the pGL3-Control. Data express the mean ± standard deviation (n=3). The nucleotide sequence of the Ca^{2+} responsive element is shown above the luciferase constructs. The consensus binding sites for the transcriptional factors AP2, MAF and c-Ets are underlined.

cells. However, 1 day after stimulation, the levels of hST3O/N mRNA decreased, and remained constant down to the time point in 2 days (Fig. 1C, upper panel). Ca^{2+} exerted a stronger influence on hST3O/N in NHK cells than PHK16 cells. hST3O and hST3Gal II mRNA expressions were not detected by Northern blot analysis in either NHK or PHK16 cells (Fig. 1 C). These results indicate that the hST3O/N gene was down-regulated during differentiation of PHK16 and NHK cells, suggesting that the hST3O/N gene is responsible for the down regulation of sialic acid that occurs during keratinocyte differentiation.

Analysis of the transcriptional starting sites on hST3O/N mRNA in the PHK16 cell line. To clarify the transcriptional regulation of the hST3O/N gene, we analyzed the transcriptional starting sites of the hST3O/N gene in PHK 16 cells, using 5′-RACE analysis. In human placenta, the cDNAs of hST3O/N consist of five isoforms, namely, types A1, A2, B1, B2, and B3 (17). Sasaki et al. (13) has also cloned type B1 form cDNA from a human melanoma cell line. In the present study 5′-RACE analysis of the NHK and PHK16 cells resulted in major extension products of 250 and 210 bp that is dependent on the presence of target cDNA during PCR (data not shown). After subcloning the PCR products and sequencing individual clones, we found two types of type B isoforms, type B3 and type BX, in the NHK and PHK16 cells. Type BX was a novel isoform related to the type B form but which differed upstream of the B3 exon (Fig. 2A). High homology (90.0 %) was observed between the 5′-untranslated region of the BX isoform and the mouse α2,3sialyltransferase gene (mST4) (35). In order to determine the hST3O/N mRNA isoforms responsible for differentiation, Northern blots containing mRNAs from PHK16 cells treated with high concentrations (1.0 mM) of Ca^{2+} were probed with a type BX-specific fragment (Fig. 2B). The levels of expression of BX mRNA from Ca^{2+} treated PHK16 cells was similar to that of non-treated PHK16 cells. The same results were obtained using NHK cells. These results suggest that the type B3 isoform is responsible for differentiation in PHK16 and NHK cells.

A differentiation responsive element exists within the hST3O/N promoter. In order to identify the element that response to differentiation, we made luciferase constructs carrying the 5′-deleted hST3O/N promoter (Fig. 3). These luciferase plasmids were then transfected into PHK16 cells. In the low Ca^{2+} condition, extension of the 5′-deletion of the hST3O/N promoter to nt 420 (pGL-420B3) reduced expression to approximately 40 % of that of the promoter with deletion to nt 520 (pGL-520B3). However, under high Ca^{2+} conditions, no difference between pGL-520B3 and pGL-420B3 was observed. This result suggests that the nt 520-420 region acts as a proximal enhancer in PHK16 cells under low, but not high, concentrations of Ca^{2+}. To determine whether the nt 520-420 region functions as a differentiation responsive element, two different reporter plasmids, in which the luciferase-gene is transcribed under control of a heterologous (SV40)-promoter, were prepared (Fig. 4). The nt 520-420 fragment was ligated in front of the SV40 promoter in both orientations. The luciferase activities of pGLPRO520-420 and pGLPRO420-520 were 2-4 fold higher than the activity of pGLPRO under low Ca^{2+} conditions, but not under high concentration of Ca^{2+}. These results indicate that the nt 520-420 region acts as a transcriptional enhancer element of the hST3O/N B3 promoter in undifferentiated human keratinocyte cells. Expression of the keratin K5 and K14 genes is high in undifferentiated basal cells and suppresses further differentiation (26), which is similar to the case of sialic acid linked to Galβ1,3GalNAc. Fig. 4 shows the nucleotide sequence of the differentiation responsive element and putative transcriptional factor binding sites. The differentiation responsive element contains two sequence similar to AP2 (36), one sequence similar to the mammary cell activating factor (MAF) recognition element (37) and one sequence similar to c-Ets (38). AP2 binding sites exist in the 5′-upstream regions of K5 and K14 gene, and AP2 is more abundant in nuclear extracts of keratinocytes than in extracts of other cell types (28). Table 1 shows the AP2-like sequences in the K5 and hST3O/N promoters. The 5′-flanking region of the B3 promoter contains eight sequence motifs ($AP2^1$-$AP2^8$) similar to the AP2 binding site (Fig. 3). The $AP2^8$ and $AP2^7$ sequences which are present in our differentiation responsive element, show high homologies with the K5 AP2 binding site. These findings suggest that the $AP2^7$ and $AP2^8$ sites mediate undifferentiated keratinocyte specific expression of the hST3O/N gene. ESE-1 is an epithelium-specific c-Ets family transcription factor (25). ESE-1 expression is induced during terminal differentiation of human keratinocyte induction with Ca^{2+} (20). Anther possible hypothesis is that the ESE-1 acts as a transcriptional suppressor of hST3O/N gene expression. Regulation of the hST3O/N gene may be mediated by specific interaction of AP2 and ESE-1 with the DNA element identified in the present study. These cellular factors may be important for keratinocyte differentiation.

TABLE 1

Comparison of AP2-like Binding Sequences

Motif	Sequence	% Identity
AP2 consensus	5′ CCCCAGGC 3′	
K5	−100 CCCCCAGGC −92	
hST30/N $AP2^8$	−489 CCCCCAGGG −481	89
$AP2^7$	−504 CCCTCAGGA −496	78
$AP2^1$	−1200 GGCCCTGGC −1192	66
$AP2^2$	−1131 TTCCCATGC −1123	66
$AP2^3$	−913 GCCCCACCC −905	66
$AP2^4$	−900 TCCCCACCC −892	66
$AP2^5$	−611 TCCCGAGTC −603	66
$AP2^6$	−605 GTCCCTGGC −597	66

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