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REGULATION OF INVOLUCRIN GENE EXPRESSION BY CALCIUM IN NORMAL HUMAN KERATINOCYTES.

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ABSTRACT.

Calcium is essential for normal epidermal differentiation. Data from Northern and nuclear run-on analysis indicate that involucrin gene transcription is induced by 1.2 mM extracellular calcium. A 3.7 Kbp fragment of the involucrin gene, which contains 2.5 Kbp of upstream region, the transcription start site, and the first intron, was sub-cloned into the pGL3-basic luciferase reporter vector and transfected into pre-confluent normal human keratinocytes (NHK). The stimulated activities of this clone were above basal levels and was further enhanced eight-fold by 1.2 mM extracelluar calcium. The results from a series of truncation and internal deletion experiments revealed multiple calcium-independent enhancer elements between -2476 and -2131 bp of the transcription start site and a calcium-dependent element between -2131 and -2028 bp. This 103 bp fragment contains sequences of an AP-1 site (TGAGTCA), a SP-1 site (GGGCGG), and shares homology with two elements in the human keratin-1 promoter, within the regions identified as mediating the calcium responsiveness of that gene in keratinocytes. One or more of these putative elements may be involved in the calcium-dependent regulation of the involucrin gene transcription in NHK.

INTRODUCTION.

Calcium is essential for normal epidermal differentiation (1). In response to calcium, normal epidermal cells (keratinocytes) form cornified envelopes as a result of cross-linking of substrates such as involucrin by a specific membrane-bound enzyme, transglutaminase-1. Previous results from our laboratory have shown that the mRNA and protein levels of both involucrin and transglutaminase-1 were induced in a synergistic fashion by calcium and 1,25-Dihydroxyvitamin D3 in normal human keratinocytes (NHK) (2). The promoter of the involucrin gene has been previously identified (3), and a 3.7 Kbp 5' flanking region of the human involucrin gene cloned in front of a beta-galactosidase reporter has been demonstrated to be sufficient for the epidermal specific expression of the involucrin gene in transgenic mice (4).

The action of calcium on keratinocyte differentiation is exerted at multiple levels involving genomic and non-genomic events. At the genomic level, two examples can be cited. For genes that can be regulated by the cAMP response element (CRE), the activity of its binding protein (CREB) can be enhanced by calcium-dependent phosphorylation by calmodulin-dependent protein kinases (5-8). The AP-1 sites are well known to be regulated by phorbol esters by their activation of protein kinase C (PKC) (9,10). Recent reports suggest that calcium-induced c-fos and junB expression may in turn regulate the function of AP-1 sites (11,12).

MATERIALS AND METHODS.

Northern blotting.

Total RNA was prepared according to the method of Chomczynski and Sacchi (13). Twenty μg total RNA per lane was electrophoresed through 1% agarose-formaldehyde gels, stained with Acridine Orange (Sigma, St Louis, MO), and then transferred to Hybond-N^+ nylon membranes (Amersham International, Buckinghamshire, UK). The blots were hybridized with the random-primed ^{32}P-labeled cDNA probe PI-2 for involucrin (a gift form Dr. Howard Green, Harvard Medical School, Boston, MA). The amount of involucrin mRNA was quantified by laser densitometry of the resulting autoradiogram.

Nuclear run-on assay.

In vitro transcription studies were performed according to established methods (14,15). Cells were washed with phosphate buffered saline and lysed in a buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA and 40% glycerol. The nuclei was then separated by centrifugation and stored in liquid nitrogen until use. Nuclei were thawed on ice and placed in an equal volume of 2X transcription solution (10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.3 M KCl, 1 mM ATP, CTP, GTP, and 200 mCi of ^{32}P was then made up to 1 mM $CaCl_2$ and incubated with RNase-free DNase (100 μg/mL) for 5 min. at room temperature. The reaction mixture was then treated with proteinase K (100 μg/mL) in 1% SDS at 37^{o}C for 30 min., extracted with phenol-chloroform, and ethanol-precipitated. An equal number of counts per min. was

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hybridized for 48 hrs at 42^{o}C to the Hybond-N^{+} (Amersham International, Buckinghamshire, UK) membrane-immobilized pGEM-3Z plasmids containing the involucrin cDNA, cDNA of glyceraldhyde-3-phosphate dehydrogenase (GAPDH), and 18S RNA. pGEM-3Z vector alone was used as a negative control for this assay.

Plasmids Constructs and DNA Sequencing.

We obtained the beta-galactosidase reporter vector that contains 3.7 Kbp of the involucrin gene from Dr. Joseph Carroll, State University of New York, Stony Brook, NY. The 3.7 Kbp fragment of the involucrin gene was sub-cloned from the beta-galactosidase vector (pNassbPL-2) to pGL3-basic to produce a construct with the involucrin promoter in front of a luciferase reporter gene.

pGL3-basic (Promega, Madison, WI) is the parent vector for all the described involucrin constructs in this report. It contains a multiple cloning site in front of the luciferase reporter gene. This vector does not contain any promoter or enhancer and was used as a negative control for all the transfections. The pGL3-control vector is similar to the basic vector except that SV40 enhancer and promoter elements have been incorporated into the vector to increase reporter activity. This vector is used as a positive control for all transfection studies. As a control for transfection efficiency, the vector pRSVbgal, which contains a beta-galactosidase gene that is driven by a Rous Sarcoma Virus promoter and enhancer, was co-transfected with each construct.

Deletion constructs B, C, A-1880, A-979, C-979, A-797, A-156, D, and F were constructed by ligating various restriction fragments of the 3.7 kbp construct (A). Deletion mutations F-2406 and F-2198 were constructed using exonulcease III / Mung bean nuclease treatment of construct F. Oligonucleotides with sequences beginning at -2177, -2131, -2028, -1947 of the involucrin gene were synthesized and used in a polymerase chain reaction (PCR) to generate DNA necessary for the construction of the 5' deletions F-2177, F-2131, F-2028, F-1947, respectively. Specifically, DNA between -2476 and -1880 of construct F were replaced with the PCR products of these oligonucleotides to generate the respective mutations.

DNA sequencing was done by dye terminator chemistry supplied by Applied Biosystems, Foster City, CA. DyeDeoxy$^{(TM)}$ terminators and AmpliTaq® were used in a sequencing reaction whose products were then loaded onto the Applied Biosystems DNA Sequencer for automated electrophoresis and analysis.

Cell Culture and transfection.

Pre-confluent keratinocytes were prepared from cells purified from newborn foreskin as previously described (16). Briefly, foreskins were incubated in 0.25% trypsin at 4^{o}C overnight to detach the keratinocytes, and the primary cultures were grown to 70-80% confluence in keratinocyte growth medium (KGM, Clonetics, San Diego, CA) containing 0.07 mM $CaCl_2$ Second passage keratinocytes were used in all experiments. The second passage NHK were plated in fresh serum-free KGM with 0.03 mM $CaCl_2$ at a density of 3 X 10^5 cells per 60 mm polystyrene dish (Nunc, Roskilde, Danmark). After 16 hours, the cells were transfected by incubating with fresh medium containing a luciferase reporter plasmid, the pRSVbgal control plasmid, and polybrene (Aldrich, Milwaukee, WI) in a platform rocker at 34^{o}C for 6 hours (modified from reference (17)). Two μg of each luciferase reporter construct and 0.2 μg of pRSVbgal were co-transfected into each dish of cells. The cells were then exposed to 2 mL of 10% glycerol in KGM for 3 minutes at room temperature and washed twice in 5 mL calcium and magnesium free phosphate buffer saline (PBS) at room temperature before replacing with fresh medium containing 0.03 mM $CaCl_2$ for an additional 16 hours. To determine the effect of calcium on luciferase reporter activity, the medium was either left untreated or supplemented with $CaCl_2$ to a final concentration of 1.2 mM for 24 hrs, and harvested as described below. Cells transfected with up to 4 μg of each plasmid DNA showed a linear increase in their respective reporter activities. High amounts of pRSVbgal DNA (1 μg or more) decreased luciferase reporter activity. When 0.3 μg or less pRSVbgal DNA was used, the inhibitory effect of the pRSVbgal DNA on luciferase activity was not detectable; therefore, a reduced amount of this vector (0.2 μg) was used in all experiments.

Reporter assays.

The transiently transfected NHK were harvested 48 hrs after transfection (24 hrs after calcium treatment). Before the harvest, the NHK were washed twice with 5 mL PBS (4^{o}C) and lysed in 400 mL "reporter lysis buffer" (Promega, Madison, WI) per dish. These cells were then detached from the plate with a cell scraper (Fisher Scientific, Pittsburg, PA), and the lysed cell debris was then removed by centrifugation (2 min. at 14,000 X g). A 10 mL aliquot of each cell extract was used to assay for either beta-galactosidase or luciferase activity using AMPGD (Tropix, Bedford, MA) or luciferin (Promega, Madison, WI), respectively, as substrates. The light output generated by each substrate was quantitated by a luminometer (GEM Biomedical, Inc., Hamden, CT). The relative transcriptional activity of each construct is expressed as the ratio of luciferase

activity to beta-galactosidase activity, and normalized to the activity ratio of the 3.7 Kbp construct cultured in 0.03 mM $CaCl_2$.

RESULTS.

Calcium treatment (1.2 mM) did not substantially affect the luciferase activity of the vectors pGL3-basic, pGL3-control, or pRSVbgal. Pre-confluent NHK were transfected with each vector and were either maintained in 0.03 mM calcium or switched to 1.2 mM calcium for 24 hours. The luminometer readings in relative light units (RLU) of cells were listed in Table 1. The variability was most likely due to difference in transfection efficiency, which range from 8 to 15%. Mock transfected cells showed light output less than 2-fold above the assay blank. Cells transfected with the pGL3-basic vector showed low luciferase activities compare to the pGL3-control vector, but were 10 to 15-fold higher than the mock transfected cell extract. Involucrin mRNA levels were low in the pre-confluent cells maintained at 0.03 mM calcium. Pre-confluent NHK cultured in 0.03 mM $CaCl_2$ were supplemented with $CaCl_2$ to a final concentration of 0.07, 0.1, 0.3, and 1.2 mM for 24 hrs. This experiment was performed a total of three times and a representative gel and northern blot are shown in figure 1. In all cases, calcium increased the involucrin mRNA levels in a dose-dependent manner with the maximum effect at 1.2 mM $CaCl_2$.

To determine if the effect of $CaCl_2$ on the involucrin mRNA level was due to increased transcription, nuclear run-on assays were performed on cells cultured at 0.03 mM $CaCl_2$ and cells incubated with 1.2 mM $CaCl_2$ for 24 hrs. Newly synthesized mRNA was labeled with ^{32}P-UTP and hybridized to immobilized pGEM-3Z plasmids containing the involucrin cDNA, GAPDH cDNA, 18S RNA cDNA, and vector alone without insert. Non-specific targets hybridized to the membrane were removed by successive washes, and the resulting blot was exposed to film. This experiments were carried out three times and a representative experiment is shown in figure 2. Densitometry of the autoradiogram indicates that the transcription rate of the cells treated with 1.2 mM $CaCl_2$ is several-fold higher than the rate of synthesis in the untreated cells (n=3). The calcium induced increase in involucrin mRNA synthesis was specific; neither GAPDH nor 18S RNA synthesis was affected by calcium. The pGEM-3Z vector, as expected, did not bind the newly formed transcripts.

To determine if the increase in mRNA transcription was due to a genomic element in the involucrin promoter, a 3.7 Kbp fragment of the involucrin gene (3,4) was sub-cloned into the pGL3-basic luciferase reporter vector. This 3.7 Kbp fragment contains 2476 bp of 5' upstream region, the first exon of 43 bp that contains a non-coding region of the involucrin mRNA, and the first intron of 1188 bp (Figure 3). All the coding sequence of the involucrin gene is located in the second exon and is not part of the 3.7 Kbp construct. Sequence analysis revealed one CRE site at -2441 and five putative AP-1 sites. Two of the AP-1 sites have been shown to be regulated by phorbol esters (18) and are noted on figure 3.

NHK transfected with the 3.7 Kbp involucrin construct (Figure 4, construct A) exhibited an 8-fold induction of the luciferase/beta-galactosidase activity ratio when incubated with medium containing 1.2 mM $CaCl_2$ for 24 hours, compared to cells that were maintained in medium with 0.03 mM $CaCl_2$. Deletion of the first intron of the human involucrin gene at +186 (B) and -3 (C) did not affect the calcium-dependence but did enhance the basal luciferase reporter activity (Figure 4). Thus, intron A contains a suppressor element of basal activity but no calcium-responsive element. A series of 5' deletion mutations using unique restriction sites within the involucrin gene at -1880, -976, -797, and -156 was then constructed and tested (Figure 4). In all these constructs, minimal reporter activities were detected, regardless of calcium concentration indicating that the region between -2476 and -1880 of the human involucrin promoter is required for basal and calcium-induced transcriptional activity.

Results with these constructs prompted the construction of two internal deletion mutations with deletions from -1880 to -156 (D) and from -1000 to -156 (E). Since the reporter activity of the construct with the deletion between -1880 to -156 (D) was responsive to calcium, a calcium-responsive element must be located between -2476 and -1880 (Figure 4). Furthermore, deletion of -1880 to -156 enhanced the basal activity by 34-fold. On the other hand, no enhancement was observed when only -1000 to -156 was removed, indicating the presence of a suppressor element between -1880 and -1000.

Since the region between -1000 and -156 and the first intron did not show calcium dependence, these regions were removed from the original involucrin promoter construct (A) to yield construct F (Figure 4). The calcium responsive region between -2476 and -1880 was then examined by a series of 5' deletions. The results of deletions at -2406, -2198, -2177, -2131, -2028, and -1947 are shown in figure 5. Between -2476 and -2131, each of the four deletions caused successive reductions in basal transcription activity, while the transcription of each could still be stimulated by calcium. Therefore, multiple calcium-independent enhancer elements exist in this region. While deletion of -2131 to -2028 had no apparent effect on the basal activity, it reduced the calcium-

Table 1. The Effects of Calcium on Beta-galactosidase and Luciferase Reporter Activities.

Vector DNA	0.03 mM Calcium (RLU ± SEM)	1.2 mM Calcium (RLU ± SEM)
pGL3-basic	247 ± 59	358 ± 55
pGL3-control	151,000 ± 24,000	145,000 ± 16,000
pRSVbgal	123,000 ± 14,000	166,000 ± 61,000

Figure 1.(Page 20 Upper) Effects of calcium on involucrin mRNA expression in NHK. NHK were initially cultured in 0.03 mM calcium. The concentration of calcium was switched to the indicated calcium concentration for 24 hours before the cells were harvested. Twenty μg total RNA was fractionated in a 1% agarose gel, transferred onto nylon membrane and probed with ^{32}P random primed-labeled involucrin cDNA probe. The blot was quantitated by densitometry. Data were normalized to the amount of the involucrin mRNA in the cells maintained in 0.03 mM calcium. Acridine Orange staining of the 28S and the 18S RNA is shown above the northern blot for the involucrin mRNA.

Figure 2. (Page 20 Lower) Nuclear run-on assay of NHK. NHK were cultured in Clonetics KGM supplemented with 0.03 or 1.2 mM calcium in the presence of ^{32}P-UTP. The newly synthesized RNA was then hybridized to pGEM-3Z containing the involucrin cDNA, GAPDH cDNA, and cDNA of 18S RNA as described in methods. The vector pGEM-3Z was used as the negative control.

Figure 3. (Page 21 Upper) The 3.7 Kbp human involucrin promoter in pGL3-basic vector. The involucrin promoter was cloned in front of the luciferase reporter gene in pGL3-basic vector. The 3.7 Kbp of the involucrin gene contains 2476 bp of the 5' untranscribed region, the first exon, and the first intron. The locations of the CRE site, the proximal and distal AP-1 sites, the SP-1 site, the TATA box, and the transcription start site are shown.

Figure 4. (Page 21 Lower) Deletion analysis of the 3.7 kbp involucrin promoter. The content of the deletion constructs is shown on the left, and their activity is shown on the right. Luciferase and beta-galactosidase were measured in lysates of keratinocytes transfected with the involucrin promoter sub-cloned into the pGL3-basic vector. The average ratios of their activities ± standard error of the mean (SEM) from at least 3 determinations were normalized with the average activity of construct A in cells cultured in 0.03 mM calcium. Where the error was less than the thickness of the line, no error bar is indicated. The open bars indicate activity ratios for cells maintained at 0.03 mM calcium, whereas the filled bars indicate the activity ratios for cells switched to 1.2 mM calcium for 24 h before they were harvested. The pGL3-basic vector without any insert was used as negative control (Vector). Construct A contains the sequence of the involucrin promoter between -2476 and +1228, and constructs B and C are 3' deletions of this construct at -182 and +3, respectively. A-1880, A-976, A-797, and A-156 were made from construct A, by removal of the 5' end of the base number indicated at unique restriction enzyme sites. Similarly, removal of the sequence between -2476 and -976 of the construct C resulted in construct C-976. Constructs D and E have internal deletions from -1000 to -156 and from -1880 to -156, respectively.

Figure 5. (Page 22 Upper) Deletions analysis of 5' flanking region of the involucrin promoter. Luciferase and beta-galactosidase activities were measured as described in the legend to figure 4. The cells were transfected with construct F and 5' truncation mutations F-2406, F-2198, F-2177, F-2131, F-2028, and F-1947. Note the difference in scale between figures 4 and 5.

Figure 6. (Page 22 Lower) Partial DNA sequence of the human involucrin promoter. AP-1 and SP-1 sites are enclosed by boxes. The direct repeat motif of GGCAGA is underlined. Regions of human keratin-1 that are homologous to the human involucrin promoter are in bold, and the locations of these sites are indicated. Locations of the 5' end of the construct F-2431 and F-2028 are also indicated.

Calcium regulation of Involucrin gene.

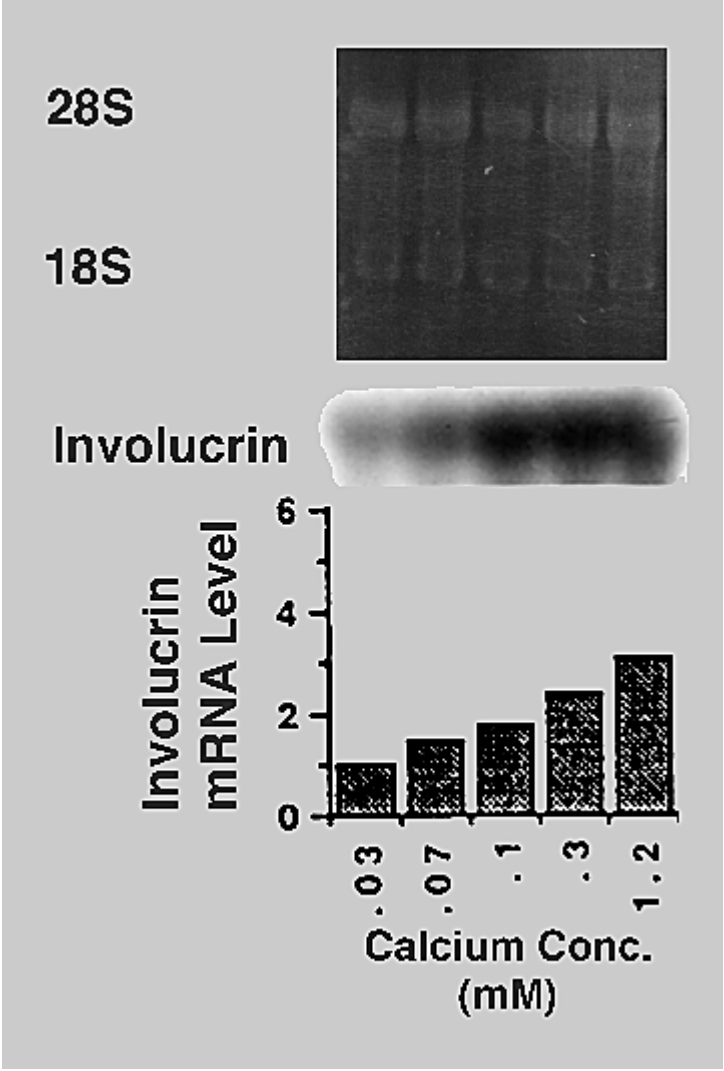

Figure 1. Effects of calcium on involucrin mRNA expression in NHK.

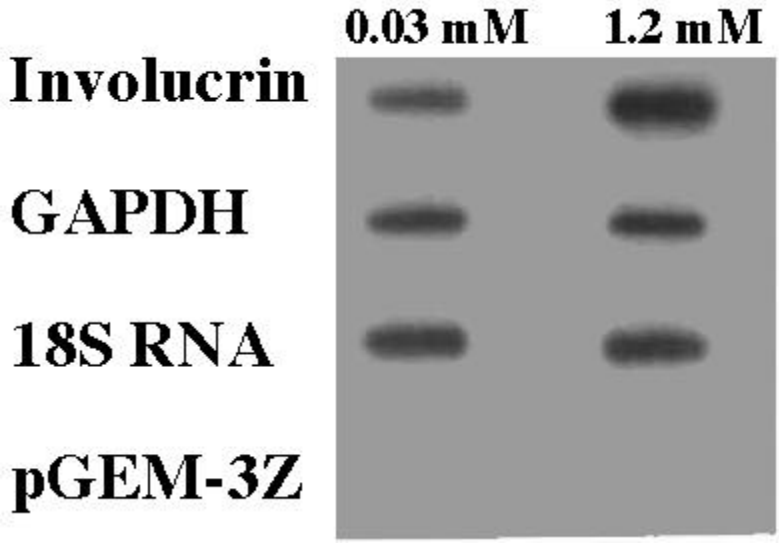

Figure 2. Nuclear run-on assay of NHK.

Calcium regulation of Involucrin gene.

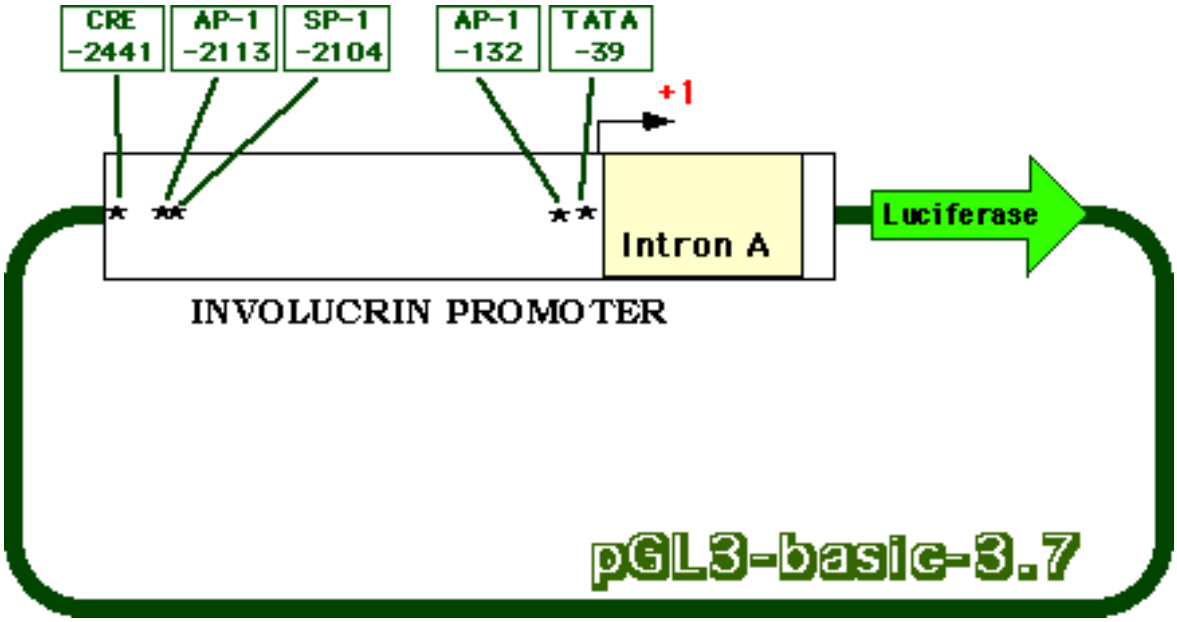

Figure 3. The 3.7 Kbp human involucrin promoter in pGL3-basic vector.

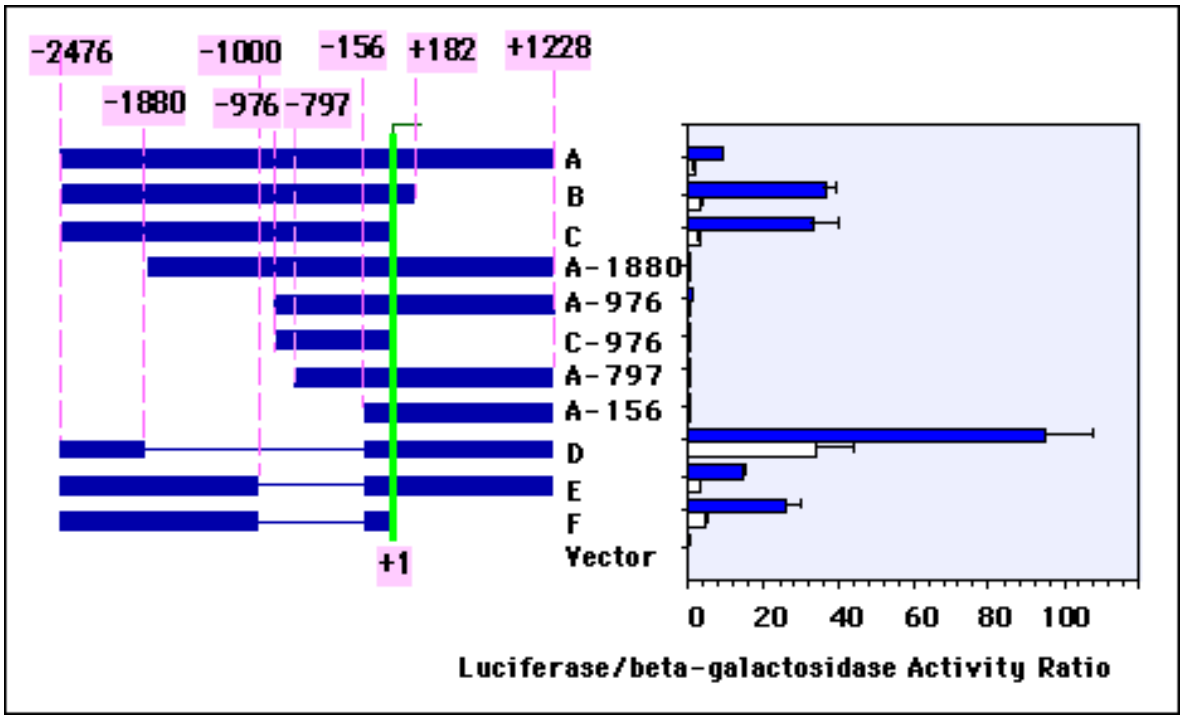

Figure 4. Deletion analysis of the 3.7 kbp involucrin promoter.

Calcium regulation of Involucrin gene.

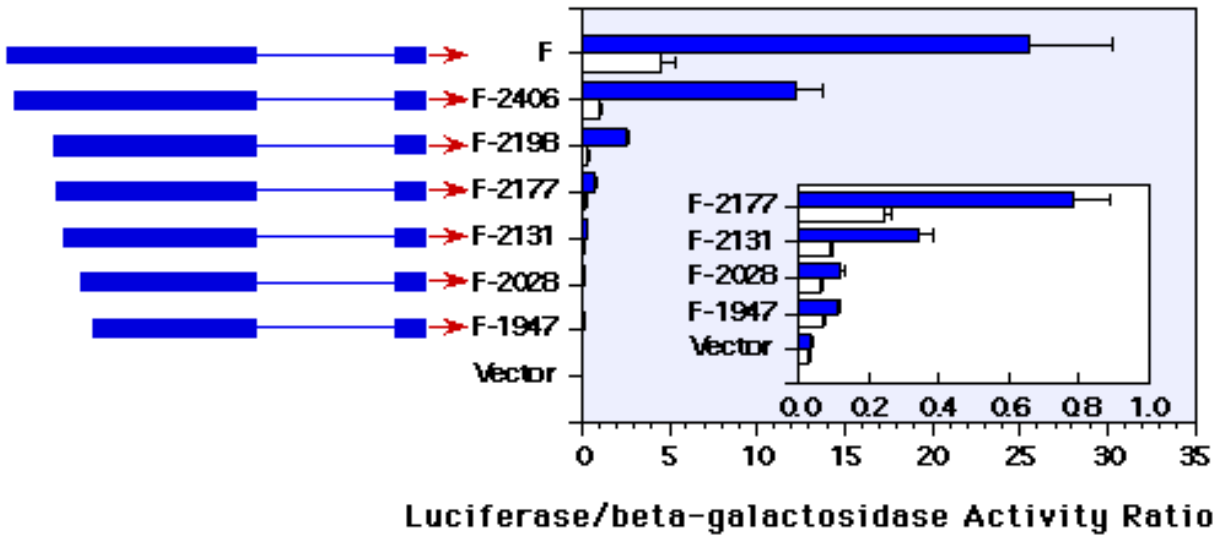

Figure 5. Deletions analysis of 5' flanking region of the involucrin promoter.

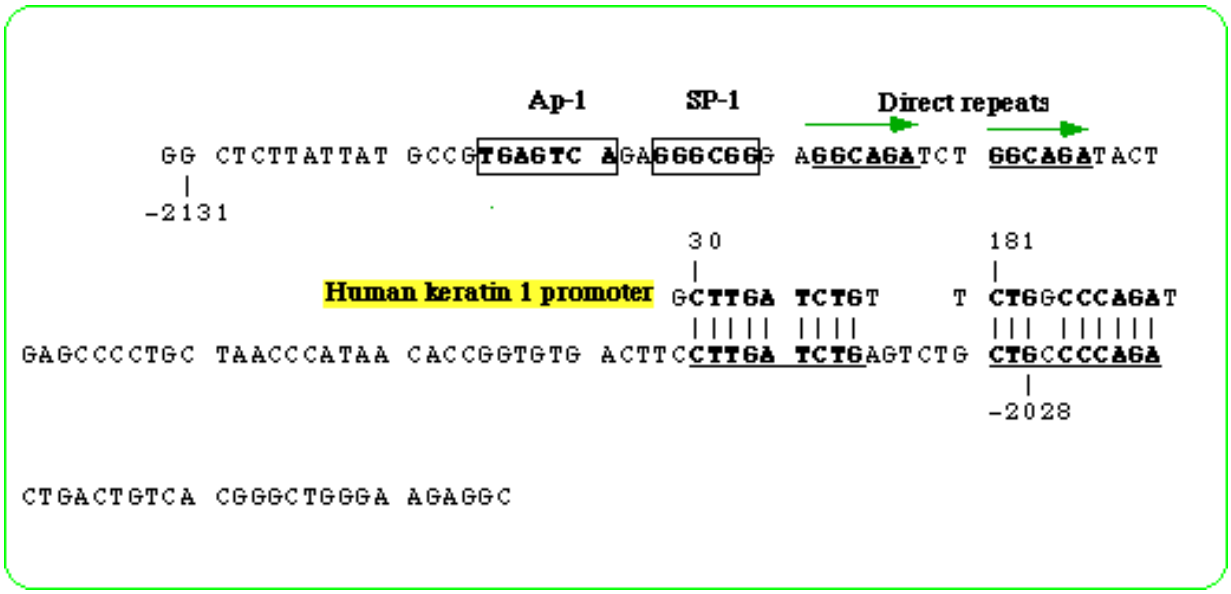

Figure 6. Partial DNA sequence of the human involucrin promoter.

induced activity greatly. Thus a calcium-dependent element is present in this region of 103 bp.

The sequence of this 103 bp region is shown in Figure 6. The sequence revealed an AP-1 site (-2116 to -2110), a SP-1 site (-2107 to -2102), a novel direct repeat (-2099 to -2085), and two regions that share homology with a 422 bp region of the human keratin-1 3' flanking region that has been previously demonstrated to contain calcium-dependent elements (19). The sequence of the first region (CTTGATCTG) is identical to the base number 20 to 38 of the human keratin-1 3' region. The second region (GTGCCCCAGA) has a 90% homology to a different region of the human keratin-1 gene (position 181 to 190).

DISCUSSION.

Extracellular calcium increases involucrin mRNA levels in NHK in a dose-dependent manner. Results from nuclear run-on experiments indicate that calcium increases the transcription rate of the involucrin gene. Thus, the involucrin gene in NHK is transcriptionally regulated by extracellular calcium.

Deletion studies using unique restriction enzymes to remove large fragments of DNA from the involucrin promoter revealed the presence of repressor elements in the region between -1880 and -1000 and in the first intron. This approach also demonstrated the existence of a calcium-dependent element between -2476 and -1880. More detailed study of this region with additional deletions refined the location of the calcium-dependent element to a region of 103 bp from -2131 to -2028.

A putative AP-1 element (-2116 to -2110) is present within this region. The AP-1 element was first shown to be inducible by phorbol esters (9,10). This element has also been demonstrated to be regulated indirectly by cAMP and calcium in neural and endocrine cells (11,12). Therefore, calcium may induce expression of the involucrin gene through the AP-1 site. In addition, there is a SP-1 site (-2107 to -2102) and a novel direct repeat (GGCAGA NNN GGCAGA) that may also play a role in either the basal or calcium-dependent transcription of the involucrin gene.

Keratin-1 gene expression is also known to be up-regulated by extracellular calcium. A region of 422 bp of the human keratin-1 promoter has been shown to contain a calcium-dependent element (19-21). Within this region, there is an AP-1 site and two regions that are homologous to the human involucrin promoter (FIGURE 6). Since both genes are induced by calcium, one or both of these sites may be involved in mediating the effect of calcium on gene transcription.

The mechanism for calcium-dependent regulation of the CRE is well known and involves CREB and Ca^{2+}/Calmodulin-dependent protein kinase II and IV (5-8). The DNA sequence of the involucrin promoter reveals a putative CRE at -2441. However, deletions of this CRE as in the F-2402 construct did not reduce the calcium-dependent transcription of the reporter gene. Thus, this CRE appears not to be important for the calcium-dependent regulation of the involucrin gene. However, deletions of the region between -2474 and -2402 in this construct did cause a 75% decrease in the basal reporter activity, suggesting that the CRE may play a role in the basal reporter activity.

In conclusion, calcium induces involucrin mRNA transcription in NHK. Calcium can also induce luciferase reporter activity in a construct driven by a 3.7 fragment of the human involucrin gene. This effect is mediated by a calcium-responsive element within a region of the involucrin gene likely to be important for calcium-dependent regulation of the involucrin gene during epidermal differentiation.

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