Involucrin is a major protein of the cornified envelope of keratinocytes that provides much of the structural integrity of the skin. The gene expression of this differentiation marker is induced by elevated extracellular calcium in cultured human keratinocytes. A 3.7-kilobase fragment of this gene contains the necessary elements to drive a luciferase reporter in a calcium-dependent manner. We have sequenced the upstream region of the involucrin promoter and localized a calcium response element that contains an activating protein-1 (AP-1) site (TGAGTCA). Mutation of this site abolished the promoter activation by calcium. Compared with cells grown in 0.05 mM calcium, the binding activity of factors within nuclear extracts from keratinocytes for this AP-1 site was enhanced 3-fold in cells grown in 1.2 mM calcium. Immunoelectrophoretic mobility shift (supershift) assays identified JunD, Fra1, and Fra2 as the major factors that bind to the AP-1 element. Western analysis of the proteins in the nuclear extracts showed that the levels of c-Jun, JunB, JunD, FosB, and Fra2 increased and the levels of c-Fos and Fra1 decreased slightly with calcium treatment. The effect of calcium on the involucrin promoter was enhanced synergistically by phorbol 12-myristate 13-acetate (PMA) in a protein kinase-dependent manner. In conclusion, calcium-regulated involucrin gene expression is mediated at least in part by AP-1 transcription factors.

The skin is a dynamic organ composed of a dermal and an epidermal layer. The epidermis can be divided morphologically into four layers (1). The basal layer contains the actively proliferating cells. As the keratinocytes migrate from the basal layer outward, numerous differentiation markers, such as involucrin (INV) (2), transglutaminase (3), loricrin (4), keratins K1 and K10 (5), and filaggrin (6), become apparent in the spinous and granular layers. The fully differentiated cells then form the cornified layer of the stratum corneum. In the outer layers of the epidermis, the cornified envelope is formed beneath the plasma membrane and is the product of extensive cross-linking of proteins such as INV, filaggrin, loricrin, small proline-rich proteins (7, 8), elafin (9), periplakin (10), envoplakin (11), and cystatin A (12) by transglutaminase and sulfhydryl oxidase.

INV is one of the major proteins that is cross-linked by transglutaminase to form the cornified envelope. A 5.8-kilobase (kb) genomic fragment of the INV gene (ATCC 61047) contains two exons, of 43 and 2107 bp, respectively, an intron of 1188 bp, and 2461 bp of 5′-upstream DNA (2). A 3.7-kb fragment of this genomic clone, which does not contain the coding region in the second exon, was subcloned into a β-galactosidase reporter vector, introduced into transgenic mice, and shown to be expressed only in the epidermis, indicating that this fragment contains the necessary signals to direct tissue-specific expression (13).

Coincident with the higher expressions of both the INV and transglutaminase genes is an increase in the intracellular calcium level (14). Extracellular calcium raises the intracellular calcium (15) and stimulates keratinocyte differentiation (16). The mRNA levels of both INV and transglutaminase are induced by elevated calcium in the culturing medium (17, 18). The increase in mRNA level is due at least in part to an increase in transcription; therefore, calcium is important for the transcriptional regulation of differentiation markers such as INV.

In this report, we have characterized the INV gene for the presence of calcium-dependent elements. We found that an AP-1 site in the upstream region of the INV gene is essential for the calcium response.

**EXPERIMENTAL PROCEDURES**

**Materials**—The vector pGL3-basic (Promega, Madison, WI) contains the luciferase reporter gene but is not linked to any promoter. The reporter in pGL3-promoter (Promega) is regulated by the SV40 promoter, and the reporter in pGL3-control (Promega) is regulated by the SV40 enhancer and promoter. DNA used for transfection and DNA sequence determination was propagated in *Escherichia coli* JM109 and was prepared using Qiagen Maxi-prep columns (Chatsworth, CA) according to the manufacturer’s protocol. Oligonucleotides were made by the Biomolecular Resource Center at the University of California, San Francisco, or Cruachem (Dullus, VA).

PKC inhibitor Ro31-8220 (Calbiochem, La Jolla, CA) was dissolved in Me2SO before being added to the cells. Stock solutions of phorbol 12-myristate 13-acetate (PMA, Sigma) were prepared in ethanol. Calcium chloride was from Fisher Scientific (Tustin, CA), and calcium-free keratinocyte growth medium (KGM) was obtained from Clonetics (San Diego, CA). Calcium- and magnesium-free phosphate-buffered saline (PBS) was obtained from Life Technologies, Inc. All other chemicals were purchased from either Fisher Scientific or Sigma.

**DNA Constructs and Mutations**—Construct INV-I was made by subcloning a 3.7-kb fragment that includes −2461 to +1228 bp of the INV gene from the H3700-pl2 (13) into the XhoI and HindIII sites of the pGL3-basic vector. The numbering of the constructs and the INV promoter were based on the location of the 5′-ends relative to the transcription start site (+1) of the INV promoter. The 3.7-kb INV promoter

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was digested with NotI and SaI enzymes. The NotI overhang of the insert and the HindIII overhang of the vector were filled in using T4 DNA polymerase and ligated to each other. The SaI overhang of INV was ligated to the compatible end of the XhoI of the vector. Truncation mutations −1890, −976, −797, and −156 from the 5’ direction, and +1803 to +2382 from the 3’ direction, were generated from the 3′ truncated construct. The truncation mutations were made by first digesting MluI+PstI/MII and XhoI, respectively. The truncation mutations were then ligated to construct INV-I. The location of each deletion site was determined by DNA sequencing.

The sequence of the PCR-generated region was confirmed by an oligonucleotide made by annealing the oligonucleotides 5′-TCATAGGATCCG-3’ and 5′-AGCTGGATCCCT-3′. A BamHI site (underlined) was introduced to facilitate screening of this construct. The resultant construct contained −2461 to −3 bp of the INV promoter. Constructs Δ−1006:−156 and Δ−1880:−156 were made by removing the −1006 to −156 bp and −1880 to −156 bp, respectively, from construct INV-I using PstI and ApaI enzymes. The PstI site is at −1880 bp and the ApaI sites are at −1006 and −156 bp. Construct INV-II was made by removing the −1006 to −156 bp DNA fragment from the Δ−3 construct using the ApaI enzyme. Construct INV-II+3′ was made by digesting the 3′ end of INV-I, −106 bp from the 3′ end of the vector, and ligating to each other. The PCR-generated region was confirmed by automated nucleotide sequencing using fluorescence dye terminators.

**PCR Mutagenesis**—The mutations −2180, −2130, −2026, and −1946 were prepared by PCR. The upstream primers used correspond to the 5′ end of the indicated positions of the INV gene. Constructs −2121 and M1 were made using oligonucleotides 5′-TCATAGGATCCGAGTCACAGAGGGC-3′ and 5′-TCATATGATCCCTGAGCGAGGACC-3′, respectively, as upstream primers. The AP-1 site is underlined and the single base pair mutation of M1 is in lowercase. The downstream primer used was designed to hybridize with −1890 to −1790 of the INV gene downstream from the PstI site (−1880). A 10-ng template DNA construct INV-I was first denatured 5 min in 10 μl of water at 94 °C under oil. An equal volume of a premixed solution was added to each tube so that the final reaction mixture contained 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl2, 0.01% (v/v) gelatin, 0.2 mM each of dATP, dGTP, dCTP, dTTP (Promega), 1 μM of each primer, 1 unit of Taq-Exender (CLONTECH, Palo Alto, CA), and 1 unit of Ampli-Taq DNA polymerase (Perkin-Elmer, Foster City, CA) in a thermocycler (Japan) in a touchdown PCR strategy. The temperature template was amplified for 30 cycles using the sequence 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. At the end of the last cycle, the reactions were further incubated at 72 °C for 6 min. PCR products were cut with PstI and subcloned into the Smal and PstI sites of construct INV-II. The DNA sequence of the PCR-generated region of each construct was confirmed by automated nucleotide sequencing using fluorescence dye terminators.

**Transfection of Human Keratinocytes**—The procedure for Polybrene-mediated transfection of primary human keratinocytes was modified from previously described protocols (18, 19). Briefly, second passage human keratinocytes were cultured from neonatal foreskins (20) and plated to a density of 500,000 cells per 60-mm culture dish in calcium-free KGM with 0.03 mM calcium. The cell suspension was pipetted in 1 ml of KGM supplemented with 0.03 mM calcium for 6 h at 37 °C in 5% CO2 on a rocker mixer. The cells were then treated with 2 ml of 10% trypsin for 3 min, washed twice with PBS (5 ml per plate), and incubated in 5 ml of KGM supplemented with 0.03 mM calcium for 16 h. Transfected cells were grown in the next day with calcium and PMA.

Each construct was tested in triplicates with two different cell preparations.

**β-Galactosidase and Luciferase Activity Measurements**—Cells were washed with PBS and lysed in reporter lysis buffer (Promega) per the manufacturer’s instructions. Aliquots (10 μl) of each cell extract were assayed for β-galactosidase activity using the substrate 4-methylumbelliferone (Promega) and β-galactosidase substrate 4-methylumbelliferyl-β-D-galactopyranoside (Tropix, Bedford, MA). The luciferase activities reported were standardized with the β-galactosidase activities and normalized to the mean activity of cells transfected with the luciferase construct INV-I, which were grown in KGM with 0.03 mM calcium.

**Sequence Analysis**—The nucleotide sequence of the 581 bp between −2461 and −1880 bp of the INV-I construct was determined in both directions using an automatic sequencer employing fluorescent dye terminators by the Biomedical Research Center at the University of California, San Francisco.
containing 0.03 mM calcium for 16 h and were transfected with pRSVgαI and construct INV-I, a luciferase reporter construct containing 3.7 kb of the INV promoter. The medium calcium concentration was then increased up to 2.4 mM, as indicated. Cells were harvested 24 h after calcium treatment, and the luciferase and β-galactosidase activities of the cell extracts were determined. Luciferase activities shown are the ratios of luciferase activities to the β-galactosidase activities, relative to the mean activity ratio of construct INV-I in keratinocytes kept at 0.03 mM calcium. Data are expressed as mean activity ratio ± SD of triplicate transfections. The arrow labeled “+1” indicates the location of the transcription start site.

Fig. 1. Dose-dependent activation of the INV promoter by calcium. Preconfluent keratinocytes were seeded and grown in KGM containing 0.03 mM calcium for 16 h and were transfected with pRSVgαI and construct INV-I, a luciferase reporter construct containing 3.7 kb of the INV promoter. The medium calcium concentration was then increased up to 2.4 mM, as indicated. Cells were harvested 24 h after calcium treatment, and the luciferase and β-galactosidase activities of the cell extracts were determined. Luciferase activities shown are the ratios of luciferase activities to the β-galactosidase activities, relative to the mean activity ratio of construct INV-I in keratinocytes kept at 0.03 mM calcium. Data are expressed as mean activity ratio ± SD of triplicate transfections. The arrow labeled “+1” indicates the location of the transcription start site.

The INV mRNA level of the keratinocytes was highest 24 h after calcium treatment. Thus, luciferase activity of the reporter construct was measured at this time, which was the earliest time point that gave a consistent and significant increase in promoter activity. Reporter activity was highest at 0.7 mM calcium and remained high at 1.2 and 2.4 mM calcium (Fig. 1). These higher doses of calcium increased promoter activity, with 1.2 mM calcium giving the most consistent result. Thus, calcium concentrations of 0.03 and 1.2 mM calcium were used in subsequent experiments to study the effects of calcium on the modified fragments of the INV gene.

Repressor and Enhancer Regions—A repressor region was identified by 3′-deletions of the INV promoter. Deletion of either +182 to +1228 bp or -3 to +1228 bp enhanced the calcium-induced and basal activities 3-fold (Fig. 2A); however, the -fold induction caused by calcium was not significantly affected. Thus, Exon 1 (+1 to +43 bp) and the intron (+44 to +1188 bp) appear to contain one or more repressors but no calcium-dependent cis element.

The 5′-end of the INV promoter was characterized by making deletion constructs −1880, −976, −797, and −156. Although the full-length construct (INV-I) showed calcium-dependent activation of the luciferase gene of 13-fold (Fig. 2A), the four deletion constructs that do not have the 5′-most region between −2461 and −1880 lost their basal and calcium-induced promoter activities (Fig. 2B). Thus, this 581-bp region appears to contain both calcium-dependent and calcium-independent enhancer elements.

To confirm the enhancer activity of the 581-bp region, internal deletions of two regions were made (Fig. 2C). Deletions of the region between −1006 and −156 bp did not significantly change the reporter activity of cells grown in either 0.03 or 1.2 mM calcium. Therefore, this region does not contain any major basal or calcium-dependent element. However, removal of −1880 to −156 bp enhanced the basal reporter activity 30-fold, indicating the presence of a strong repressor between −1880 and −1006 bp. However, the effect of calcium on the reporter activities was reduced from 13- to 3-fold. Although the reduction in calcium-dependent activation may be due to limitation in the ability of calcium to further stimulate such high basal activity, these results suggest the presence of an enhancer element in this region that is important for the calcium-dependent activation of the INV gene.

The 581-bp Upstream Region Contains a Calcium Response Element—To optimize the calcium induction, construct INV-II containing both calcium response regions (−2461 to −1880 and −1880 to −1006) and the 156-bp proximal promoter was made so that the dominant calcium response region between −2461 and −1880 bp could be analyzed. Elevated calcium in the medium induced reporter activities of this construct 3- to 8-fold.

DNA sequence of the 581-bp upstream region is shown in Fig. 3A. Two 5′-deletion constructs (−2409 and −2196) were made by exonuclease III/mung bean digestion. These deletions reduced both the basal and calcium-induced activity, but the activities of these constructs remained dependent on calcium (Fig. 3B). Additional constructs with the 5′-end beginning at −1880 to −156 bp showed a reduction in reporter activity.

Fig. 2. Localization of the calcium-response region in the INV promoter. A, construct INV-I contains −2461 to +1228 bp of the INV gene. Luciferase activities were determined in extracts of keratinocytes transfected with 3′-truncated constructs of the INV promoter. Constructs “+182” and “−3” are 3′-deletions of INV-I. Cells were either left untreated (0.03 mM calcium, open bar) or supplemented with calcium to a final concentration of 1.2 mM (filled bar) for 24 h. See Fig. 1 for unit definition. Data are expressed as mean activity ratio ± S.D. of triplicate transfections of two independent clones. B, the numbering of the constructs is based on the location of the 5′-ends relative to the transcription start site (+1) of INV. C, in constructs Δ−1006: −156 and INV-II, the DNA within −1006 to −156 bp was removed from construct INV-I and “−3,” respectively. In construct Δ−1880: −156, DNA corresponding to −1880 to −156 bp was removed from construct INV-I.
Fig. 3. Nucleotide sequence and deletion analysis of the INV upstream region. A, the nucleotide sequence of INV genomic DNA of the region between -2461 and -1881 bp is shown in the box. The sequence corresponding to the oligonucleotides used in the EMSA in Figs. 5 and 8 is underlined. Black bars indicate the location of the consensus AP-1 and the stimulating protein-1 (SP-1) sites. B, INV-II contains -2461 to -1006 bp of the upstream region and -156 and -3 bp of the INV promoter (see Fig. 2C). The location of the 5'-end of each construct is indicated on the left of the bar graph. Involucrin promoter activity is as defined in Fig. 1.

-2180, -2130, -2026, and -1946 bp were made using a PCR-based method. Deletion constructs -2180 and -2130 maintained their calcium response; however, the calcium response was lost with further deletions of the 5'-end (Fig. 3B). The locations of the 5'-ends of all mutants and the nucleotide sequences of the PCR-generated fragments were confirmed with DNA sequencing.

We observed large changes in the activity of the reporter constructs. The variation in activity ratio is a reflection of the changes in basal activity seen with successive deletions. Thus, the 3.7-kb construct was used as a reference in all the experiments.

Examination of the region between -2130 and -2026 bp revealed an AP-1 site at -2114 to -2108 bp. To test the role of the AP-1 site in the calcium responsiveness, double-stranded oligonucleotides containing the AP-1 site were introduced into construct -2026 in both the forward (For) and reverse (Rev) orientations at the SalI site of the pGL3 vector, 2 kb 3' to the INV transcription start site. The AP-1 site of the INV gene appeared to be functional in both orientations (Fig. 4) in that the calcium responsiveness of the “For” and “Rev” constructs were restored to the level of the -2130 construct. Truncation of the -2130 construct to -2121 bp (construct -2121) did not affect the reporter activity, but changing the T to a C at -2110 bp in the corresponding construct (M1) abolished its calcium inducibility to the level of the -2026 construct. Therefore, the AP-1 site at -2114 to -2108 bp is functional in keratinocytes and is essential for the calcium-induced promoter activity.

AP-1 Proteins in Nuclear Extracts of Keratinocytes—To confirm the binding ability of this AP-1 site, oligonucleotides containing the INV AP-1 site were used as probes in EMSA. Nuclear extracts of the cells grown in 0.03 and 1.2 mM calcium were analyzed. Addition of nuclear extracts to the AP-1 oligonucleotide resulted in two shifted bands (Fig. 5A). The intensity of these bands was three times higher in the gel lane, where nuclear extracts from cells grown in 1.2 mM calcium was used, than in the nuclear extracts from cells grown in 0.03 mM calcium, suggesting that the AP-1 binding factors were elevated in cells grown in 1.2 mM calcium. The top band appeared to be a result of specific binding of nuclear factors to the AP-1 site, because addition of homologous oligonucleotides completely abolished their binding to the probe. The lower band was less specific, in that competition by the unlabeled probe was incomplete. Addition of double stranded oligonucleotides containing a 5-bp substitution of the AP-1 site (M5) resulted in no competition, although addition of AP-1 oligonucleotides with...
a 1-bp substitution (M1) resulted in a modest inhibition of binding to the upper band.

We then tested whether the two different mutations had different effects on involucrin promoter activity. The distal AP-1 element as well as the M1 and M5 mutations of this element were subcloned into a reporter construct that contained the proximal 156 bp of the INV promoter. Although the original AP-1 sequence enhanced the calcium dependence over 2-fold, both the M1 and M5 eliminated this activity (Fig. 5A). Thus, even though it had lost its ability to activate the INV promoter in a calcium-dependent manner, the M1 mutation retained some ability to bind to nuclear proteins in vitro.

Synergistic Effects of PMA and Calcium Actions—Because the AP-1 site also mediates the effects of phorbol esters, we evaluated the interactions between PMA and calcium on the INV gene expression. INV-I was transfected into keratinocytes, pretreated with 1 μM calcium, and, at 8, 16, 24, 32, 40, 48, and 56 h before harvest, 1.2 μM calcium or 10 nM PMA was added to the cells. Near-maximal calcium response was reached by 32 h, and the promoter activity remained elevated for at least 56 h (data not shown). PMA activation of the INV gene reached near-maximal levels by 16 h and remained elevated for at least 40 h. Although both increased the INV promoter activity, their time courses of action were different. When they were added separately, calcium and PMA enhanced reporter activities 5- and 41-fold, respectively (Fig. 6). When cells were treated with both agents, the promoter activity was induced synergistically to 118-fold. The effects of calcium and PMA were abolished by pretreatment of the cells with 1 μM of a potent PKC inhibitor Ro31-8220. Therefore, protein kinase C (PKC) is likely to be involved in both the calcium and PMA activation of the INV promoter.

Requirement of the Proximal INV Promoter for the Calcium-dependent AP-1 Activity—Single base pair mutation of the AP-1 site in INV-I and INV-II resulted in loss of promoter activity (data not shown); therefore, we investigated the function of the AP-1 site in shorter constructs. We tested the effectiveness of the intact (AP-1) or 5-bp mutation (mut) of the distal INV AP-1 element (−2114 to −2108 bp) on the SV40 promoter, a 116-bp INV promoter (INV-116) that does not contain the proximal AP-1 site, and a 156-bp INV promoter (INV-156) that contains the proximal AP-1 site. Calcium enhanced the SV40 promoter and the 116-bp INV promoter 2-fold, but stimulated the 156-bp INV promoter 7-fold (Fig. 7A). The calcium effect was lost when the distal INV AP-1 element was mutated. Thus, these results suggest that both the distal and the proximal AP-1 sites are necessary for calcium regulation of the INV gene.

The regulation by calcium of the INV promoter is different from that by PMA (Fig. 7B). Introduction of the distal AP-1 site to the SV40 promoter of pGL3-basic enhanced the PMA-dependent promoter activity by 2-fold similar to that of calcium. However, this AP-1 element increased the PMA-dependent activation of the INV 116-bp promoter activity by 13-fold. In the presence of the INV 156-bp promoter, PMA increased the promoter activity by 72-fold. Like the calcium effect on these constructs, the PMA effect on the promoter activity was reduced when constructs containing the mutant forms of the distal AP-1 site were used. Because removal of the region between −156 and −116 in the INV 156-bp promoter also reduced the -fold induction of the promoter activity, both the proximal and the distal AP-1 sites appear to be required for optimal PMA effect. However, unlike calcium, PMA could stimulate promoter activity substantially with only one AP-1 site.

Effects of Calcium and PMA on the AP-1 Factors—Calcium and PMA appear to have different effects on the levels of the AP-1 proteins. The factors in the nuclear extracts that bind to the AP-1 element were identified using antibodies to c-Jun, JunB, JunD, c-Fos, FosB, Fra1, and Fra2. Antibodies made for JunD, Fra1, and Fra2 produced slower migrating bands, indicating that these proteins are present in the DNA-protein complex observed by EMSA (Fig. 8). Nuclear extracts of PMA-treated cells were also analyzed by EMSA. Due to the low intensity of the EMSA bands in the PMA-treated nuclear extracts, the identification of the specific proteins by supershift may have been hindered. As a result, only Fra1 is readily identifiable in this nuclear extract.

Western analyses of the nuclear extracts of keratinocytes showed the presence of c-Jun, JunB, JunD, c-Fos, FosB, Fra1, and Fra2 in keratinocytes (Fig. 9). Rabbit IgG gave no visible band in our Western blots. Antibodies raised against c-Jun and JunB identified multiple bands in the keratinocyes. All but one of the multiple bands detected by c-Jun and JunB antibodies can be eliminated by preabsorption of the antiserum by corresponding peptides. The exception is the top band seen in the c-Jun Western blot, which was only partially absorbed with c-Jun peptides. Thus, the higher molecular mass bands may be aggregate forms of the AP-1 proteins. In all other cases, each antibody yielded a single dominant band between 40 and 47 kDa. Calcium treatment for 48 h resulted in an increase in
levels of the 151- and 55-kDa bands of c-Jun, 53- and 40-kDa bands of JunB, a 42-kDa band of JunD, a 46-kDa band of FosB, and a 47-kDa band of Fra2. The levels of the 40-kDa band of c-Fos and the 45-kDa band of Fra1 were reduced slightly by calcium. The 105- and 42-kDa bands of c-Jun, the 97- and 34-kDa bands of JunB and a 97-kDa band of c-Fos remained unchanged.

The Western data shown in Fig. 9 are representative of data from three different primary keratinocyte cultures. The levels of most AP-1 proteins as detected by specific antibodies are reproducible. We have, however, noticed elevation in the basal levels of junD and FosB in the nuclear extract from one of these keratinocyte cultures, in which case the induction by calcium and PMA was less apparent. It is unclear what the basis of the variation may be, but differences in the source of the primary keratinocyte cultures may have contributed to this variation.

Calcium and PMA treatments appear to have different effects on the AP-1 proteins. The intensities of most of the c-Jun bands decreased with 24 h of PMA treatment. The only exception is that the 55-kDa band is more intense than that of the vehicle-treated cells. The results for the JunB bands are mixed. The 40-kDa band of JunB increased with PMA treatment, but the 53- and 82-kDa bands decreased with treatment. The 42-kDa band of JunD, the 40-kDa band of c-Fos, the 45-kDa band of Fra1, and the 47-kDa band of Fra2 all increased with PMA treatment, whereas the FosB band was unchanged.

Treatments with PMA and calcium caused additional increases in intensities of the 34- and 40-kDa bands of JunB and the 47-kDa band of Fra2. In the cases of JunD, c-Fos, FosB, and Fra2, the additional calcium treatments did not change the levels of these proteins as compared with cells that had only the PMA treatment. In summary, JunB, JunD, and Fra2 appear to be increased by both calcium and PMA, whereas the same agents appear to have different effects on the levels of c-Jun, c-Fos, FosB, and Fra1.

Nuclear extracts from cells treated with calcium for 24 h were also analyzed by EMSA, supershift, and Western blotting. No qualitative difference was observed between nuclear extracts from cells grown in 1.2 mM calcium for 24 h compared with that from cells grown in 1.2 mM calcium for 48 h (data not shown).
DISCUSSION

The expression of the INV mRNA in human keratinocytes is induced by calcium in a time- and dose-dependent manner (17, 23, 24). Results from nuclear run-on assays indicate that calcium increases INV mRNA transcription. The fold induction of the reporter activities is more than that observed for the endogenous mRNA. The difference in the stability of luciferase and the INV mRNA may have contributed to such results. The context of the INV promoter in the reporter constructs versus that of the native genome also may have contributed to this difference. Similar to that of endogenous mRNAs, dose-dependent increases by calcium in reporter activity were observed when the 3.7-kb genomic fragment of the INV gene (construct INV-I) was used to drive the luciferase promoter in keratinocytes. Thus, the 3.7-kb fragment contains the necessary transcription elements to allow calcium induction of the INV promoter.

By a series of truncation and internal deletions, we have localized a calcium response element within a 581-bp region of the INV gene. This region contains a DNA sequence that is important for directing the tissue-specific expression of the INV gene in keratinocytes. Our sequence of this region deviates slightly from a previously reported sequence of the human INV gene (25). However, except for one nucleotide insertion, our sequence agrees well with that from a more recent report (26).

Along with the 581-bp region, elements in a 874-bp region, between −1880 and −1006 bp of the transcription start site, also appeared to modulate the calcium responsiveness of the INV promoter. A recent report identified an AP-2-like element, between −1770 and −1683 bp from the transcription start site that may be responsible for this effect (27). However, the INV AP-2-like element did not enhance the calcium-dependent promoter activity when placed in the INV-156 and INV-I Δ−1880: −156 constructs (data not shown). The function of the AP-2-like site does not appear to have direct interaction with the 581-bp calcium response region.

Deletions of different fragments of the 581-bp region suggest the presence of multiple enhancer elements. Deletion constructs −2404, −2196, −2180, and −2130 each caused a significant reduction in both the basal and calcium-induced reporter activities. Because deletion of the 104-bp fragment spanning −2130 to −2026 bp resulted in the elimination of calcium inducibility, we conclude that a calcium-dependent element is present in this fragment. Inspection of the nucleotide sequence of the 104-bp region revealed an AP-1 site at −2114 to −2108 bp. We have found that this AP-1 site is important for calcium inducibility of the INV gene, because this AP-1 site functions in a direction-independent and sequence-specific fashion to convey calcium responsiveness. In addition to the INV gene, the AP-1 element appears to be important for calcium activation of the keratin K1 gene (28, 29), and AP-1 elements are present in many keratinocyte-specific genes, including transglutaminase, loricrin, profilaggrin, and other keratins (30). The AP-1 proteins in keratinocytes may serve to control the transcription of various differentiation markers, and regulation of the levels and activities of these factors may be a crucial step in the regulation of differentiation in the keratinocytes.

The AP-1 site we identified as a calcium-dependent element was described previously as a PMA-dependent site (22), but the effects of calcium and PMA on the AP-1 site appear to differ. The time courses of stimulation by calcium and PMA were different, and, when both agents were added to the keratinocytes, synergistic activation was observed. The faster actions of PMA as compared with that of calcium suggest that some of the calcium effects may be mediated by activation of PMA-dependent pathways. Both the actions of calcium and PMA were greatly reduced by a potent PKC inhibitor, Ro31-8220, but,
Calcium and PMA extracts were then incubated with 32P-labeled double stranded DNA of rabbit IgG, anti c-Jun, JunB, JunD, c-Fos, FosB, Fra1, or Fra2. These nuclear extracts were then incubated with 32P-labeled double stranded oligonucleotides containing the INV AP-1 sequence. Top arrows (Ab-Prot-DNA) of each panel denote the bands detected by the indicated antibodies suggesting the presence of these AP-1 proteins in the protein-DNA complex.

because the inhibition by the PKC inhibitor was incomplete, some of the calcium effect may involve PKC-independent mechanisms. Therefore, although their actions may both require PKC, calcium and PMA appear to have different effects on the INV promoter activity.

Calcium action appears to require both the AP-1 site in the proximal 156 bp of the INV promoter and the distal AP-1 site at -2114 to -2108. Deletion or mutation of either the distal AP-1 site or the proximal AP-1 site in the INV-156 construct resulted in loss of calcium activation. In contrast, either the distal AP-1 site or INV-156 was sufficient to mediate the PMA action, although when both AP-1 sites were present the stimulation by PMA on the INV promoter was markedly enhanced. Thus, the context or the surrounding sequence of the AP-1 element may also play a role in the calcium responsiveness of the INV gene.

We identified JunD, Fra1, and Fra2 by EMSA as proteins that bind to the AP-1 element in cells treated with 1.2 mM calcium. Welter et al. (22) identified JunB, JunD, and Fra1 in PMA-treated cells. Under our culturing conditions, we were only able to confirm Fra1 as one of the proteins that binds to the AP-1 element after the PMA treatment. Not all the AP-1 factors induced by calcium and PMA are bound to the AP-1 element of the involucrin gene. The apparent discrepancy may be due to the inherent difference between the two methods. Although the supershift experiments identified the AP-1 factors that bind to the involucrin promoter AP-1 element under the conditions of EMSA, the Western blot analysis showed that the protein levels of the AP-1 factors in the cell regardless of their binding to the involucrin promoter. Although we were unable to use EMSA to show a qualitative difference in AP-1 factors binding to the AP-1 site following calcium and PMA stimulation, calcium and PMA may lead to altered activity of the AP-1 proteins induced through post-translational modifications such as phosphorylation.

The actions of calcium and PMA may require changes in the levels and activity of Jun and Fos members. We have demonstrated that the concentrations of some of the factors that bind to the AP-1 element were higher in cells grown in elevated calcium. The amounts of Jun and Fos proteins vary among the different layers of the human epidermis (31). In particular, c-Fos, Fra1, Fra2, and c-Jun protein levels were higher in the upper layers of the epidermis, the site of INV synthesis and increased calcium. Of these factors, we observed that the protein levels of the c-Jun and Fra2 proteins increased with calcium treatment in our in vitro model. Therefore, these factors may be responsible for the calcium induction of differentiation markers such as INV. In our cellular model, PMA, but not calcium, treatment induced c-Fos and Fra1. Thus, the increased levels of these two proteins found in the upper layers of the epidermis may be a result of a PMA-dependent signaling mechanism, such as that involving protein kinase C that is independent of calcium-signaling mechanisms.

In the cultured mouse keratinocytes, JunB, JunD, Fra1, and Fra2 were elevated with calcium treatment, and c-Jun, JunB, JunD, c-Fos, and Fra1 were induced by PMA treatment (32, 33). Although the profiles for most AP-1 proteins are similar in both the mouse and human keratinocytes, c-Jun is induced by PMA in mouse but not human keratinocytes. Fra1 is elevated...
by both PMA and calcium in mouse keratinocytes but is only inducible by PMA in human keratinocytes. In contrast, only calcium can induce the Fra2 protein level in mouse keratinocytes, whereas both calcium and PMA can increase the Fra2 protein level in human keratinocytes. The significance of the species differences is unclear. However, the growth factors in the culturing medium may have affected the activity and expression of the AP-1 factors differently and thus contributed to the differences observed between the human and mouse keratinocytes in vitro. Although calcium and PMA have slightly different effects on the same AP-1 proteins in mouse and human keratinocytes, these data support the hypothesis that different subsets of the AP-1 protein family are invoked by calcium and PMA.

Intracellular calcium stimulates c-Fos expression in many cell types, including mammosomatotrope GH3B6 cells (34), cerebellar granular neurons (35), the corticotroph cell line AtT20 (36), and Sertoli cells (37). These effects are likely to be mediated by phosphorylation of the cAMP response element binding protein by calcium/calmodulin protein kinases and activation of the cAMP response element in the c-Fos promoter (38–41). Similar mechanisms may be responsible for the change in levels of the c-Fos and, possibly, other AP-1 proteins in intracellular calcium.

INV is an essential protein in the mature epidermis, and calcium is crucial for epidermal differentiation. We have identified an upstream region that contains a number of enhancers important for optimal expression of the gene. Among them, an AP-1 element appears to be essential for the induction of the INV gene by calcium. This and other elements are likely to be required for optimal expression of the differentiation markers in the epidermis during development.

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