

# Expression of Murine Epidermal Differentiation Markers Is Tightly Regulated by Restricted Extracellular Calcium Concentrations In Vitro

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**Abstract.** Epidermal differentiation is characterized by a series of coordinated morphological and biochemical changes which result in a highly specialized, highly organized, stratified squamous epithelium. Among the specific markers expressed in differentiating epidermis are (a) two early spinous cell proteins, keratins 1 and 10 (K1 and K10); and (b) two later granular cell proteins, filaggrin and a cornified envelope precursor (CE). In vitro, epidermal basal cells are selectively cultured in 0.05 mM Ca<sup>2+</sup> medium, and terminal differentiation is induced when the Ca<sup>2+</sup> concentration is increased to 1 mM. However, only a small fraction of the cells express the markers K1, K10, CE, or filaggrin in the higher Ca<sup>2+</sup> medium. To explore the factors required for marker expression, cultured epidermal cells were exposed to intermediate Ca<sup>2+</sup> concentrations and extracts were analyzed using specific antibody and nucleic acid probes for the four markers of interest. These studies revealed that marker expression was enhanced at a restricted concentration of Ca<sup>2+</sup> in the medium of 0.10–0.16 mM. At this Ca<sup>2+</sup> concentration, both protein and mRNA levels for each marker were

substantially increased, whereas at higher or lower Ca<sup>2+</sup> concentrations they were diminished or undetected. The percentage of cells expressing each marker was increased two- to threefold in the permissive Ca<sup>2+</sup> medium as determined by immunofluorescence analysis. This optimal level of Ca<sup>2+</sup> was required both to initiate and sustain marker expression. At the permissive Ca<sup>2+</sup> concentration, expression of the markers was sequential and similar to the order of appearance in vivo. K1 was expressed within 8–12 h and K10 was expressed in the ensuing 12–24-h period. CE and filaggrin were expressed in the subsequent 24 h. Inhibition of K1 expression by cycloheximide suggested that an inducible protein was involved. Other investigators have determined that a shallow Ca<sup>2+</sup> gradient exists in epidermis, where the basal cells and spinous cells are in a Ca<sup>2+</sup> environment substantially below serum Ca<sup>2+</sup> levels. These in vitro results suggest that the Ca<sup>2+</sup> environment is a fundamental regulator of expression of epidermal differentiation markers and provide an explanation for the existence of the Ca<sup>2+</sup> gradient in vivo.

**M**AMMALIAN epidermis has been a useful model to analyze tissue differentiation. The major products of epidermal differentiation are keratin intermediate filaments. Characteristic structural differences identify two major families of keratin intermediate filaments. The type I subclass includes at least 12 proteins with molecular masses of 40–60 kD and an acidic isoelectric point; the type II subclass is composed of proteins with molecular masses of 50–70 kD and a neutral to basic isoelectric point. This classification is functionally significant since epithelial cells express type I and II keratins in specific pairs (Eichner et al., 1984; Hanukoglu and Fuchs, 1983; for review see Steinert and Roop, 1988). The predominant pair expressed in mouse basal epidermal cells is a 60-kD type II keratin (K5) and a 55-kD type I keratin (K14) (Breitkreutz et al., 1984; Roop et

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al., 1983; Schweitzer and Winter, 1983). In suprabasal cells, K5 and K14 gene expression is repressed, although the proteins persist, and synthesis of a new subset of differentiation-specific keratins, a 67-kD type II (K1) and 59-kD type I (K10), is initiated. These become the quantitatively predominant proteins of differentiated keratinocytes (Breitkreutz et al., 1984; Roop et al., 1988; Schweitzer and Winter, 1983). Synthesis of K1 precedes that of K10, and K1 expression has been detected in basal cells which have committed to differentiate (i.e., lost proliferative potential) but have not yet migrated suprabasally (Regainer et al., 1986; Schweitzer et al., 1984). Keratin expression is largely regulated at the transcriptional level (Fuchs and Green, 1979; Roop et al., 1988), although posttranslational modification of K1 in the stratum corneum has been described (Bowden et al., 1984; Steinert, 1988). A qualitatively identical process occurs in human epidermis (Eichner et al., 1986; Fuchs and Green, 1980).

In addition to keratins, maturing epidermal cells express unique proteins associated with the later stages of differentiation. Filaggrin is a basic protein of the granular cell layer and comprises the major component of keratohyaline granules (Harding and Scott, 1983; Lonsdale-Eccles et al., 1984; Steinert et al., 1981). First expressed as a high molecular weight precursor of repeated peptide units, filaggrin is processed by specific proteases to its monomeric form and interacts with keratin filaments to produce macrofibrils and tonofilament bundles (Dale et al., 1978; Harding and Scott, 1983; Lonsdale-Eccles et al., 1984; Steinert et al., 1981). The granular cell layer also is the major site for the initiation of cornified envelope formation and is rich in both the enzyme catalyzing cornification, epidermal transglutaminase, and its substrates, the cornified envelope precursors (Rice and Green, 1979; Thatcher and Rice, 1985). A number of envelope precursors have been described (Buxman et al., 1976; Kubilus et al., 1987; Rice and Green, 1979; Simon and Green, 1984; Zettergren et al., 1984) and, one, involucrin, has been cloned and sequenced (Eckert and Green, 1986; Tseng and Green, 1988). A cysteine and glycine rich protein (cornified envelope precursor protein [CE]<sup>1</sup>), which is the probable major component of the mouse epidermal cornified envelope, has recently been identified (Mehrel et al., 1988). It is transcriptionally regulated in the granular cell layer where the peptide and epidermal transglutaminase are abundant.

While numerous reports have documented the molecular changes associated with epidermal differentiation, little information is available concerning the factors which regulate this process. In cultured epidermal cells, the concentration of extracellular Ca<sup>2+</sup> (Ca<sub>o</sub>) can select for a basal or differentiated cell phenotype (Hennings et al., 1980; Yuspa, 1983). Under conditions where Ca<sub>o</sub> is <0.1 mM, keratinocytes proliferate rapidly, express a basal cell phenotype, and do not cornify, while higher Ca<sub>o</sub> induces squamous differentiation. Phorbol esters can also induce terminal differentiation in basal keratinocytes (Yuspa et al., 1982), suggesting that epidermal differentiation is linked to the activation of protein kinase C (Yuspa et al., 1983). Increasing Ca<sub>o</sub> or adding ionomycin to basal cell culture medium stimulates epidermal phosphatidylinositol metabolism to release diacylglycerol and inositol trisphosphate (Jaken and Yuspa, 1988; Tang et al., 1988). Phorbol esters act synergistically with increasing Ca<sub>o</sub> or ionomycin to accelerate differentiation (Jaken and Yuspa, 1988). Together these findings suggest that the activation of protein kinase C through a Ca<sup>2+</sup>-mediated phosphatidylinositol catabolic pathway could be a physiological regulator of epidermal differentiation (Yuspa et al., 1983). Physical analyses of tissue sections from mouse and human epidermis have demonstrated the existence of a gradient of Ca<sup>2+</sup> in which basal layer Ca<sup>2+</sup> content is extraordinarily low while granular layer Ca<sup>2+</sup> content is extremely high (Malmquist et al., 1984; Menon et al., 1985). The factors which maintain a Ca<sup>2+</sup> gradient in vivo are unknown, but their existence supports the validity of the Ca<sup>2+</sup> effect in vitro.

Ca<sup>2+</sup>-induced differentiation in vitro has been studied in detail (Yuspa, 1983). When specific keratin markers were analyzed using monospecific antisera and indirect im-

1. *Abbreviations used in this paper:* Ca<sub>o</sub>, extracellular Ca<sup>2+</sup> concentration; CE, cornified envelope precursor protein.

munofluorescence (Roop et al., 1987), K5 and K14 were expressed in basal cells in 0.05 mM Ca<sup>2+</sup> medium. K1 and K10 were not expressed in basal cells but were induced by 1 mM Ca<sup>2+</sup> medium. K1 expression always preceded K10, but only a minority of cells (<15%) in the culture population synthesized either K1 or K10, yet all of the cells underwent morphological terminal differentiation. This disassociation of a programmed cell death from expression of specific markers had also been observed in phorbol ester-treated mouse skin in which differentiation was accelerated, but the expression of K1 and K10 mRNA was reduced (Toftgard et al., 1985). Thus, the rapid acceleration of differentiation by signals such as phorbol esters or 1 mM Ca<sup>2+</sup> may have interfered with the proper integration of the signaling pathways and the coordinate expression of specific markers (Toftgard et al., 1985; Roop et al., 1987). Since a shallow Ca<sup>2+</sup> gradient has been measured in the lower epidermal layers in vivo, a gradual change in the extracellular Ca<sup>2+</sup> environment might be required for proper expression of differentiation markers. For these reasons, we have reevaluated keratinocyte responsiveness to particular concentrations of extracellular Ca<sup>2+</sup>. The results indicate that the sequential expression of epidermal differentiation markers requires specific extracellular Ca<sup>2+</sup> concentrations.

## Material and Methods

### Cell Culture

Epidermal cells from newborn BALB/c mice were isolated and cultured in Eagles medium with 8% FCS, 1% antibiotic, and a Ca<sup>2+</sup> concentration of 0.05 mM as previously described (Hennings et al., 1980). In some experiments the Mg<sup>2+</sup> concentration in the medium was increased to 4 mM to enhance growth (Tennenbaum et al., 1988). The addition of Mg<sup>2+</sup> did not influence the expression of differentiation markers (not shown). Cells were cultured at 1.5 × 10<sup>5</sup> cells/cm<sup>2</sup> in 60-mm (for immunofluorescence and Western blotting) or 150-mm tissue culture dishes (Falcon Labware, Oxford, CA) (for RNA isolation). After ~7 d, primary cultures were switched to various Ca<sup>2+</sup> concentrations as described in the individual experiments. After the appropriate time period, cells were harvested by scraping into lysis buffer for protein analysis by SDS-PAGE and immunoblotting and for RNA analysis by Northern blotting. In some experiments, cells were labeled for 3 h with [<sup>35</sup>S]methionine in methionine-free medium before harvesting to assess protein synthesis. For immunofluorescence analysis, washed cell monolayers were fixed in ethanol and processed as described previously (Roop et al., 1987). Medium Ca<sup>2+</sup> concentrations were confirmed by analysis in an atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). Biochemical assays presented are the results of determinations of extracts from single or duplicate dishes, and experiments were generally performed in duplicate and always repeated several times with similar results.

### Antisera and Immunofluorescence

Monospecific antisera to specific epidermal differentiation markers K1, K10, CE, and filaggrin were prepared in rabbits or guinea pigs by injecting unique synthetic peptides corresponding to carboxy-terminal amino acid sequences deduced from the nucleotide sequence of cDNA clones for K1, K10 (Roop et al., 1984), CE (our unpublished data), and a synthetic peptide corresponding to residues 24–39 in the partial sequence of filaggrin (Rothnagel et al., 1987). The rabbit antibodies were affinity purified using the synthetic peptide coupled to activated Sepharose (Brinkley et al., 1980). Rabbit antibodies to K1 (AF109), K10 (AF106), CE (AF62), and filaggrin (AF111) were used at 1:500, 1:1,000, 1:1,000, and 1:1,000, respectively. Monospecific guinea pig antiserum to K14 was used at 1:2,000 to double label keratinocytes. The methods for preparing dilutions and double labeling of cultured cells have been described in detail (Roop et al., 1987). Stained dishes were examined in a microscope (Labophot; Nikon Inc., Garden City, NY) using a B2 filter block for FITC and a G filter for Texas red. Multiple fields were

photographed using 10 and 20× objectives and ASA-400 daylight film. Multiple prints were counted by a neutral observer to quantitate keratinocytes by red fluorescence (K14 positive) and marker expression by yellow-orange cells (Roop et al., 1987).

### Protein Gel Electrophoresis, Blotting, and Fluorography

Proteins were extracted by two methods. For examination of cytoskeletal proteins, the method of Bowden et al. (1984) was used. Alternatively, whole-cell extracts were prepared by heating cell pellets at 100°C for 5 min directly in 10 mM Tris-HCl, pH 7.5, 5% SDS, and 20% β-mercaptoethanol. Protein control was established by the Bramhall procedure (Bramhall et al., 1969). Approximately equal amounts of protein were separated in 8.5% polyacrylamide gels in the SDS buffer and visualized by Coomassie blue staining. Protein bands were electrophoretically transferred to nitrocellulose (BA83; Schleicher & Schuell, Inc., Keene, NH) in 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol for 14 h at 60 V. Nitrocellulose blots were reacted with the same antisera used for immunofluorescence as follows: AF109, AF106, AF111, and AF62 at 1:500, 1:500, 1:200, and 1:200, respectively. Positive bands were visualized using peroxidase-conjugated second antibody and an immunoblot peroxidase assay kit (Bio-Rad Laboratories, Richmond, CA). Where indicated, radiolabeled proteins were separated by PAGE and the gels were subjected to fluorography by using EN<sup>3</sup>HANCE (New England Nuclear, Boston, MA) followed by exposure to x-ray film (X-Omat AR5, Eastman Kodak Co., Rochester, NY) at -70°C.

### Northern Blot Analysis

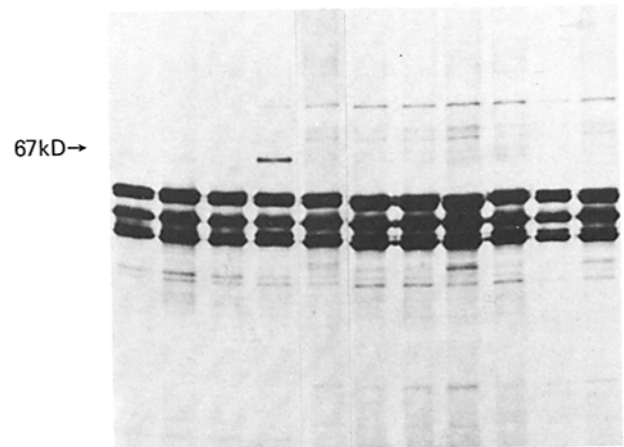
At least two 150-mM culture dishes were used to isolate RNA by the method of Chirgwin et al. (1979). Approximately 20 μg total RNA/lane was fractionated on a 1.1% agarose/formaldehyde gel and transferred to nitrocellulose (BA85; Schleicher & Schuell, Inc.) (Toftgard et al., 1985). Because of the large size of the filaggrin mRNA (17 kb), the RNA gels to be probed for filaggrin were first soaked in 0.05 mM NaOH in 1 × SSC for 20 min and rinsed twice with 10 × SSC before transfer to nitrocellulose (Alwine et al., 1977). Dried and baked filters were hybridized to specific 3' noncoding subclones of cDNAs for K1, K10, K14, CE, and filaggrin that were <sup>32</sup>P-labeled (specific activity of 2 × 10<sup>8</sup> cpm/μg DNA) by nick translation. Filters were washed to a final stringency of 0.1 × SSC, 0.1% SDS at 68°C and autoradiographed at -70°C with X-Omat AR5 film (Eastman Kodak Co.) and an intensifying screen. In some confirmatory experiments, RNA was probed as above after being transferred to nitrocellulose filters in a slot-blot apparatus.

## Results

### Specific Extracellular Ca<sup>2+</sup> Determines K1 Synthesis

The protein gel electrophoresis pattern of cytoskeletal extracts from cultured mouse keratinocytes labeled for 3 h with [<sup>35</sup>S]methionine is shown in Fig. 1. Keratins K5, K6, K14, K16, and K17 (60, 59.5, 55, 50, and 51 kD, respectively) are the predominant labeled bands and these are not modified by Ca<sub>o</sub> under standard culture conditions of low (0.05 mM) or high (1 mM) Ca<sup>2+</sup> medium (Roop et al., 1983). K1 (67 kD) synthesis is generally not detected, and K10 cannot be distinguished on a one-dimensional gel among the highly expressed bands in the 59–60-kD region (Roop et al., 1983). When basal cell cultures (0.05 mM Ca<sup>2+</sup>) are changed to 0.1 mM Ca<sup>2+</sup> medium for 24 h, a prominent 67-kD band is labeled. By 48 h in 0.1 mM Ca<sup>2+</sup>, the 67-kD band is not labeled by a [<sup>35</sup>S]methionine pulse. Other Ca<sub>o</sub> (0.5 and 1 mM) are not permissive for 67-kD labeling at any time studied. Furthermore, when cultures are medium changed stepwise through 0.1, 0.5, and 1 mM Ca<sup>2+</sup> at 24-h intervals and labeled after 48 or 72 h, the 67-kD band is not observed (not shown). Several higher molecular weight bands are labeled under all conditions of Ca<sub>o</sub> > 0.1 mM, but these are too large to be keratins (Fig. 1) and have not been identified.

Time (h)	24	48	72	24	48	24	48	72	24	48	72
Ca <sup>2+</sup> (mM)	.05	.05	.05	0.1	0.1	0.5	0.5	0.5	1.0	1.0	1.0

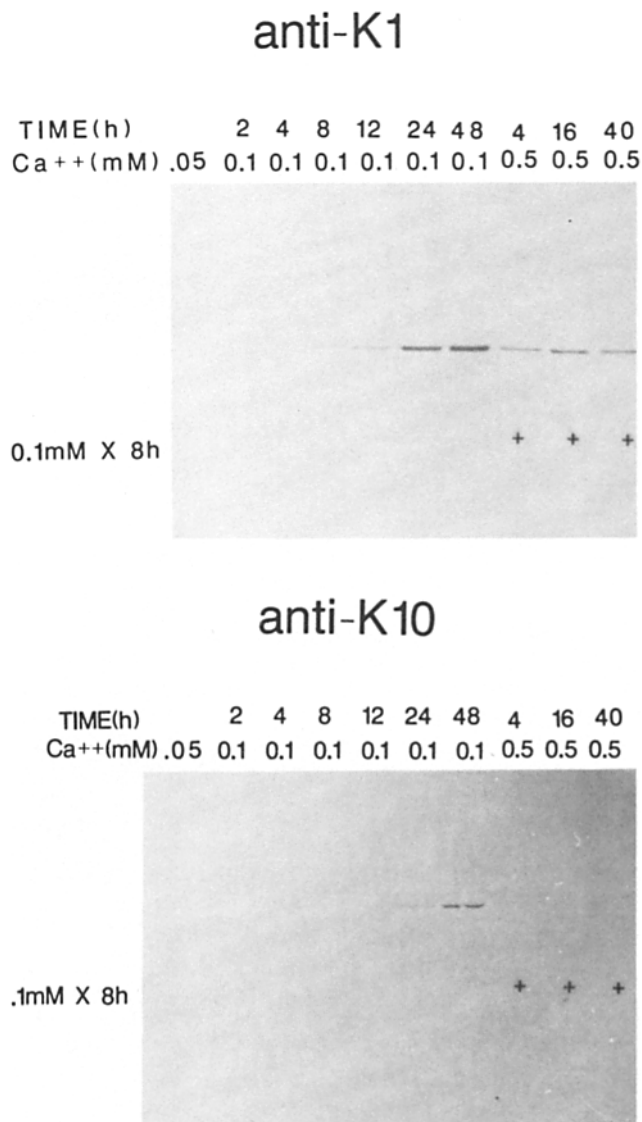


**Figure 1.** Protein gel electrophoresis of [<sup>35</sup>S]methionine-labeled cytoskeletal proteins of cultured keratinocytes. Primary epidermal cells were plated at 4 × 10<sup>6</sup> cells/60-mm culture dish in 0.05 mM Ca<sup>2+</sup> medium. Medium was changed every other day for 6 d, and, on day 7, cultures were changed to higher Ca<sup>2+</sup> medium (0.1, 0.5, or 1 mM) as indicated on the figure. 3 h before the indicated time, cultures were exposed to 85 μCi/ml [<sup>35</sup>S]methionine (1,100 Ci/mmol; New England Nuclear) in methionine-free medium containing the appropriate Ca<sup>2+</sup> concentration. Duplicate dishes were harvested, cells lysed, and cytoskeletal proteins isolated as described in Materials and Methods. Approximately equal amounts of protein were separated on 8.5% polyacrylamide gels containing 0.1% SDS and 2% β-mercaptoethanol, visualized with Coomassie blue, destained, and exposed to x-ray film for 3 d at -70°C.

These results indicate that the synthesis of the 67-kD cytoskeletal protein is dependent on a Ca<sub>o</sub> of between 0.1 and 0.5 mM and is transient.

### K1 and K10 Expression are Both Ca<sup>2+</sup> and Time Dependent

To establish that the 67-kD band was K1 and to define a time course for expression, cells were exposed to 0.1 mM Ca<sup>2+</sup> medium for varying time periods, and cytoskeletal extracts were probed for K1 content by Western blotting with monospecific anti-K1 antiserum (Fig. 2). In this experiment, anti-K1 serum did not detect a band in 0.05 mM Ca<sup>2+</sup>, but a 67-kD band was first noted at 8 h in 0.1 mM Ca<sup>2+</sup>, became more prominent by 12 h, and was intense by 24 h. Band intensity changed minimally between 24 and 48 h. When cultures were exposed to 0.1 mM Ca<sup>2+</sup> for 8 h and then changed to 0.05 mM Ca<sup>2+</sup> for additional periods encompassing 12, 24, or 48 total h in higher Ca<sup>2+</sup> medium (Fig. 2; 0.5 mM Ca<sup>2+</sup>), K1 content was always below that of cells maintained in 0.1 mM Ca<sup>2+</sup> for 24 or 48 h. This indicates that 0.1 mM Ca<sup>2+</sup> medium is required for both initiation and maintenance of K1 synthesis. When these extracts were probed for K10 content with monospecific antiserum (Fig. 2), a single K10 band was noted after 24 h in 0.1 mM Ca<sup>2+</sup>. Unlike K1, K10 band intensity increased between 24 and 48 h, suggesting that K10 expression begins later but continues longer than K1 when differentiation is induced by Ca<sup>2+</sup>. In the groups exposed to 0.1 mM Ca<sup>2+</sup> medium for



**Figure 2.** Western blot analysis to determine the time course of expression of K1 and K10 in cultured keratinocytes. Cultures were prepared as in Fig. 1 but without radiolabeling. After separation in 8.5% polyacrylamide gels, cytoskeletal proteins were electrophoretically transferred to nitrocellulose and reacted with monospecific antiserum to K1 or K10 using peroxidase-conjugated second antibody for visualization. The break in the 0.1 mM Ca<sup>2+</sup>, 48-h band in the K10 blot is an artifact. (+) Cultures exposed to 0.1 mM Ca<sup>2+</sup> for 8 h before increasing the Ca<sup>2+</sup> to 0.5 mM for the additional time indicated. The 0.05-mM group was extracted with the 24-h time points.

only 8 h and then changed to 0.5 mM Ca<sup>2+</sup> for later time points, K10 expression was not observed. This indicates that K10 synthesis was inhibited at 0.5 mM Ca<sup>2+</sup> as observed for K1.

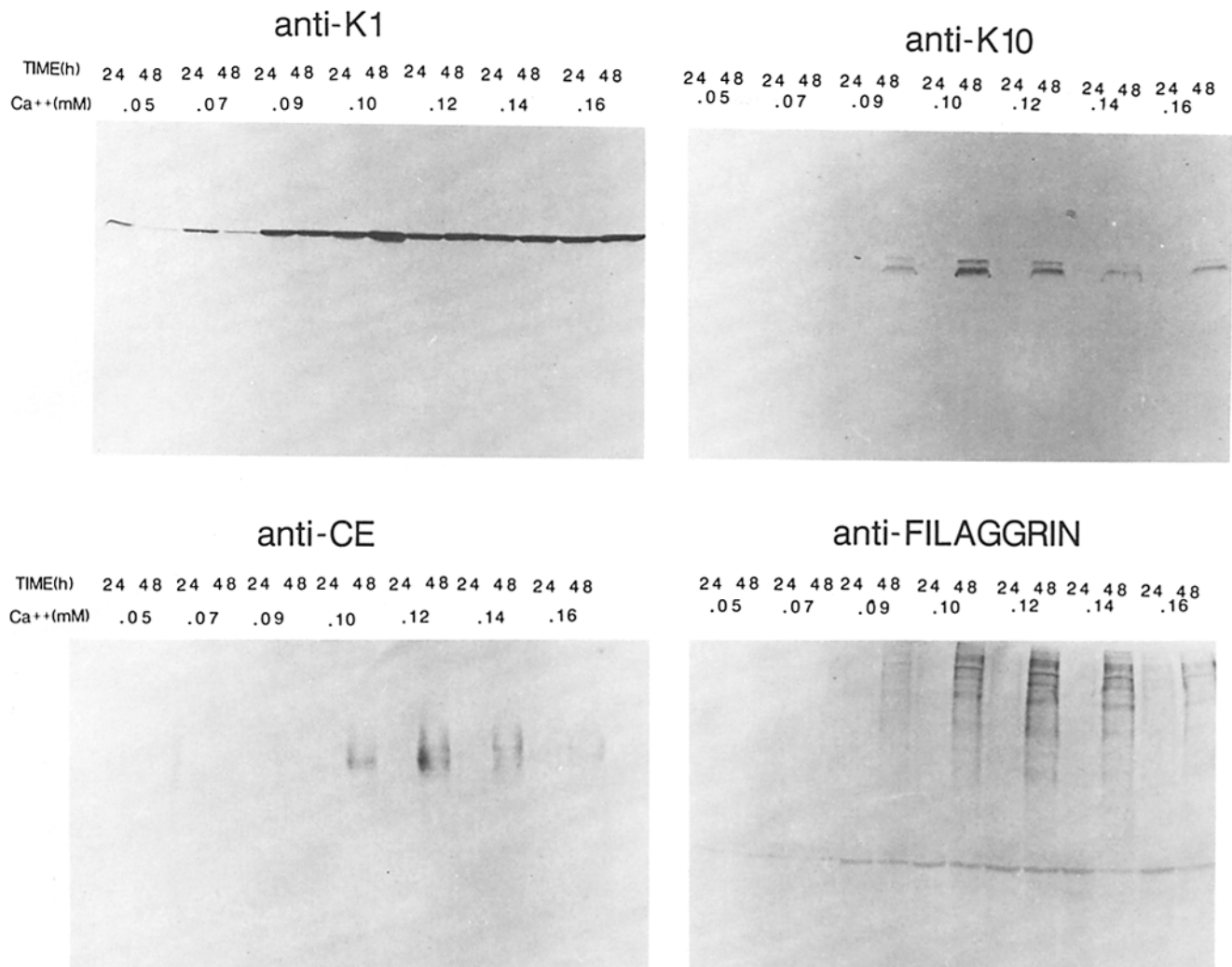
#### **Four Differentiation Markers Are Ca<sup>2+</sup> Dependent for Initiation and Maintenance of Expression**

To determine the precise Ca<sup>2+</sup> requirements for K1 and K10 as well as later differentiation markers, CE and filaggrin, basal cells were changed to a range of Ca<sub>o</sub> and extracts were studied at 24 and 48 h by blotting protein gels with

monospecific antisera. K1 was detected in cells grown in 0.05 and 0.07 mM Ca<sup>2+</sup> medium (Fig. 3) in this experiment and has been seen variably in others at these Ca<sup>2+</sup> concentrations. However, K1 band intensity increased substantially at 0.09 mM Ca<sup>2+</sup> and was most intense at 0.1 mM Ca<sup>2+</sup>. Intense K1 bands were also obvious at 0.12, 0.14, and 0.16 mM Ca<sup>2+</sup> at both 24 and 48 h. The expression of K10 (Fig. 3) was more restricted than K1. K10 was not detected in 0.05 or 0.07 mM Ca<sup>2+</sup>, expression was moderate in 0.09 mM and most intense in 0.1 mM Ca<sup>2+</sup> medium, and higher Ca<sub>o</sub> resulted in progressively lower amounts of K10. As noted previously, K10 was just detectable after 24 h and became prominent between 24 and 48 h at all Ca<sub>o</sub> > 0.9 mM. In cultured epidermal cells, K10 antisera commonly recognizes a second band that is coexpressed with K10. This additional band most likely corresponds to K11. Although the carboxy-terminal sequence of K11 has not been determined, Huszar et al. (1986) have produced a monoclonal antibody (Kk 8.60) which also detects K10 and K11 in extracts from human epidermis. It is not known whether K10 and K11 are closely related gene products or result from posttranslational modification. We have previously observed coordinate expression of K10 and K11 in other studies (Roop et al., 1988).

CE migrates as a broad band between 50–60 kD on polyacrylamide gels (our unpublished data), possibly due to its unusual amino acid composition (57% glycine, 22% serine, and 7% cystine). CE expression is tightly linked to Ca<sub>o</sub> (Fig. 3). CE was detected maximally at 0.12 mM Ca<sup>2+</sup> and was reduced at either higher or lower Ca<sub>o</sub> in the same extracts studied for K1 and K10. Filaggrin expression in vitro (Fig. 3), seen as multiple bands ranging from >200 kD to ~21 kD, was similar to the in vivo pattern where filaggrin is made as a large precursor and processed (Dale et al., 1978; Harding and Scott, 1983; Lonsdale-Eccles et al., 1984; Steinert et al., 1981). Filaggrin expression was also optimal in 0.12 mM Ca<sup>2+</sup> medium. Ca<sub>o</sub> did not affect processing of filaggrin since the band pattern was similar in all lanes. Expression of both CE and filaggrin was minimal at 24 h, but readily apparent by 48 h. The band at ~35 kD, observed with the filaggrin serum, is neither Ca<sup>2+</sup> nor time dependent and has not been seen in other experiments.

Calcium sensitivity for all four markers of differentiation was retested in experiments where a permissive Ca<sup>2+</sup> concentration of 0.1 mM was maintained for 24 h followed by a change to higher Ca<sub>o</sub> for an additional 24 h (Fig. 4). K1 is highly expressed in 0.1 mM Ca<sup>2+</sup> after 24 and 48 h. An increase in Ca<sub>o</sub> after 24 h had little effect on K1 as anticipated since its expression is virtually complete by this time. Initiating K10 (and K11) expression in 0.1 mM Ca<sup>2+</sup> for 24 h was sufficient to achieve high expression at 48 h in 0.1–0.3 mM Ca<sup>2+</sup> medium, but changing to 0.4 and 0.5 mM Ca<sup>2+</sup> reduced K10 band intensity. CE and filaggrin band intensities were similar to that of K10 with respect to Ca<sub>o</sub> sensitivity. Expression was high in 0.1–0.2 mM Ca<sup>2+</sup> for the second 24 h, but was substantially decreased in Ca<sub>o</sub> > 0.2–0.3 mM. The reduction of CE at higher Ca<sub>o</sub> could be related to a cross-linking of the protein and its removal from the pool of solubilized proteins, but this seems unlikely since maximal transglutaminase activity is extracted from keratinocytes cultured in 0.1 mM Ca<sup>2+</sup> (Lichti and Yuspa, 1988). The processing pattern of filaggrin in vitro differs from the band pattern of newborn epidermal filaggrin as shown in Fig. 4.



**Figure 3.** Western blot analysis to determine the Ca<sup>2+</sup> requirement for expression of differentiation markers in cultured keratinocytes. Primary cultures were prepared as in Fig. 1 and extracted after 24 or 48 h in the indicated Ca<sup>2+</sup> medium. Equivalent amounts of protein from total-cell lysates were separated by electrophoresis, transferred to nitrocellulose, and reacted with monospecific antibodies to K1, K10, CE, or filaggrin.

However, the newborn epidermis was exposed to trypsin during isolation and is lacking the high molecular weight filaggrin precursor probably as a result of an artifactual processing.

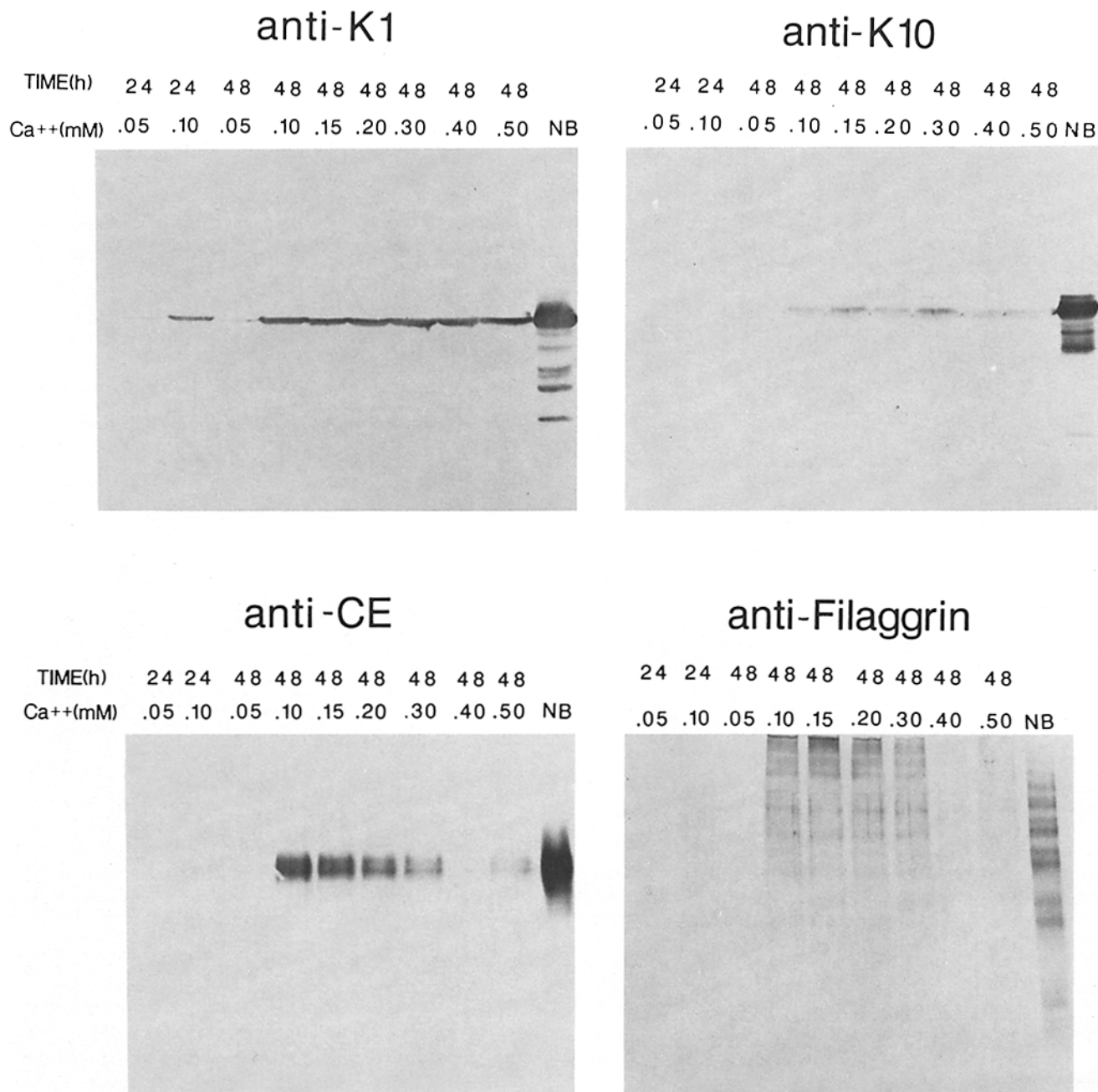
#### **Ca<sub>o</sub> Determines the Number of Cells Expressing Differentiation Markers**

Cultures exposed to differing Ca<sub>o</sub> for 48 or 72 h were processed for indirect immunofluorescence using double-labeling techniques for K14 and specific markers of differentiation. This method allows the determination of the percentage of keratinocytes in a microscopic field expressing a particular second marker (Fig. 5). In agreement with previous studies (Roop et al., 1987), marker expression was observed in a minority of cells changed to 1 mM Ca<sup>2+</sup> (11, 7, 12, and 27% for K1, K10, CE, and filaggrin, respectively; *n* = 2 determinations). When cells were changed to either 0.1 or 0.12 mM Ca<sup>2+</sup>, the number of positive cells increased two- or threefold (28, 22, 25, and 56% for K1, K10, CE, and filaggrin, respectively; *n* = 3–6 determinations). A qualita-

tive change was observed in the appearance of filaggrin granules (Fig. 5) which were larger, more intensely fluorescent, and perinuclear as well as more abundant in the 0.12 mM Ca<sup>2+</sup> cultures relative to 1 mM Ca<sup>2+</sup>. Differences among 0.1 and 0.12 mM Ca<sup>2+</sup> groups could not be documented by indirect immunofluorescence. No conditions of Ca<sub>o</sub> were found to cause all cells to express a specific marker of terminal differentiation.

#### **Ca<sub>o</sub> Regulates Marker Expression by a Transcriptional Mechanism**

Using specific cDNA probes for each marker, RNA was analyzed from cells exposed to varying Ca<sub>o</sub> for different time periods. Fig. 6 indicates that the level of transcripts for K1 and K10 is regulated by Ca<sub>o</sub> and is time dependent in correspondence to protein synthesis. The transcript for K1, detected in 0.05 mM Ca<sup>2+</sup> in this study, increased substantially after 12 h in 0.1 mM Ca<sup>2+</sup> but not in 1 mM Ca<sup>2+</sup>. K1 message remained elevated at 18 and 24 h in the permissive Ca<sub>o</sub> but not in 1 mM Ca<sup>2+</sup>. K10 mRNA was not detected in 0.05

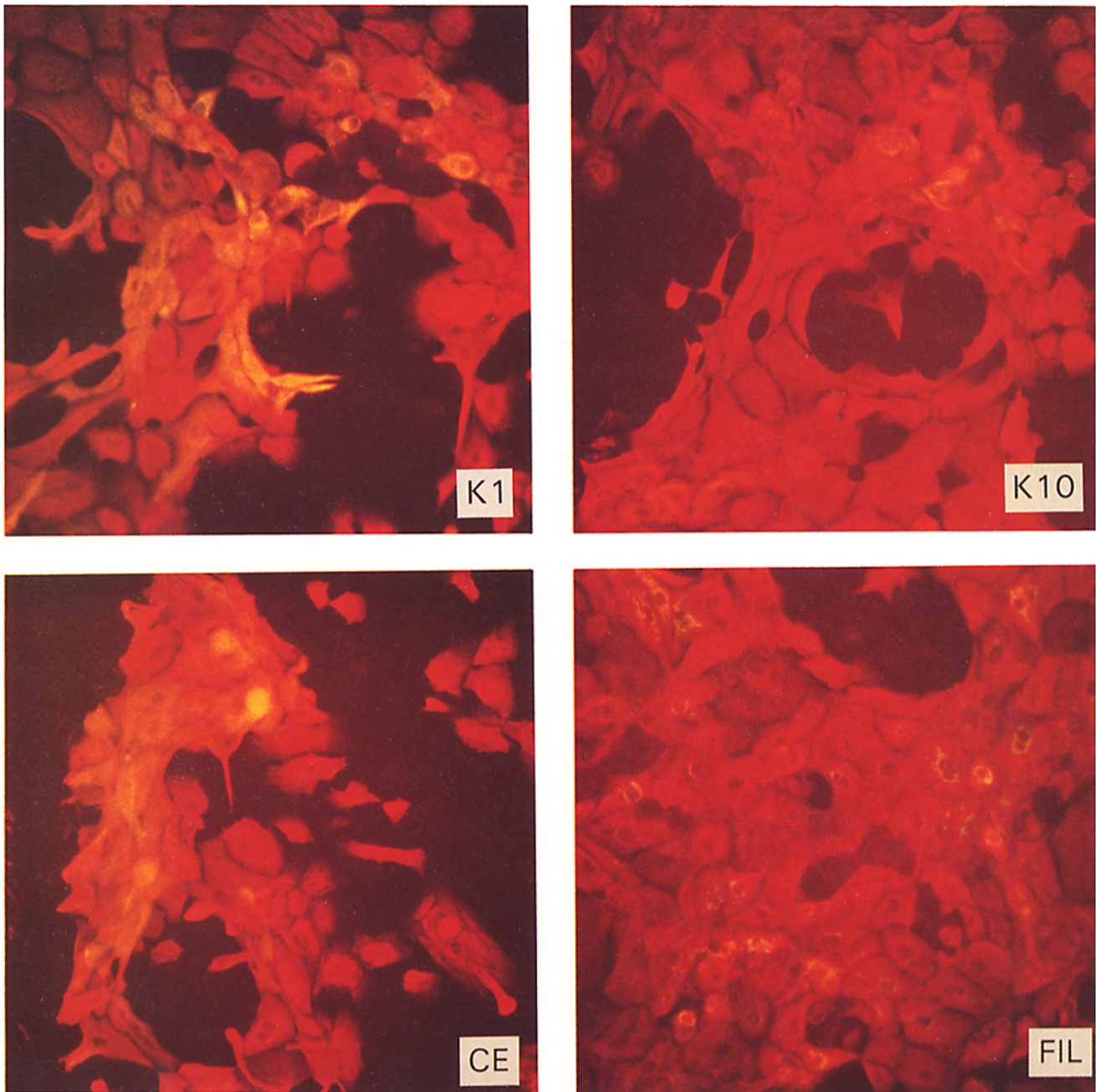


**Figure 4.** Western blot analysis to determine the Ca<sup>2+</sup> requirement for maintenance of expression of differentiation markers in cultured keratinocytes. Primary cultures were prepared as in Fig. 1 and maintained in 0.05 mM Ca<sup>2+</sup> for 6 d. In some cultures, 0.05 mM Ca<sup>2+</sup> medium was continued for 24 or 48 h, whereas other cultures received 0.1 mM Ca<sup>2+</sup> medium for 24 h followed by medium of the same or higher Ca<sup>2+</sup> concentration for an additional 24 h. Equivalent amounts of protein from total-cell lysates were separated by electrophoresis, transferred to nitrocellulose, and reacted with monospecific antisera. (Lane NB) Protein extracted from trypsin-separated (Yuspa and Harris, 1974) newborn epidermis where degradation of K1 and K10 is obvious.

or 1 mM Ca<sup>2+</sup> but was detected in cells maintained in 0.1 mM Ca<sup>2+</sup> for 24 h. Hybridization of the blots of Fig. 6 with a K14 probe indicated that the relative changes in K1 and K10 expression were not artifacts of gel loading. In vitro, the level of K14 mRNA does not change reproducibly during culture at any Ca<sub>o</sub> tested although, in vivo, K14 transcripts disappear rapidly in the first suprabasal cell layer (Roop et al., 1988). Thus, a generalized nonspecific effect of Ca<sub>o</sub> on epidermal transcription is unlikely.

The ability of a particular Ca<sub>o</sub> to modify transcript levels

in a time-dependent manner was evaluated for all four markers (Fig. 7). K1 transcripts (2.4 kb) were abundant in 0.1 mM Ca<sup>2+</sup> but not 0.5 mM Ca<sup>2+</sup> after 24 h as seen previously. Substantial reduction in this message was noted by 48 h in 0.1 mM Ca<sup>2+</sup> consistent with diminished synthesis of the protein observed in Fig. 1. The reduction in K1 transcript was similar when cultures were switched to 0.5 mM Ca<sup>2+</sup> after 24 h (Fig. 7, lane g). The abundance of K10 message (2 kb) was also increased in 0.1 mM Ca<sup>2+</sup> by 24 h but diminished between 24 and 48 h. Previously we observed an

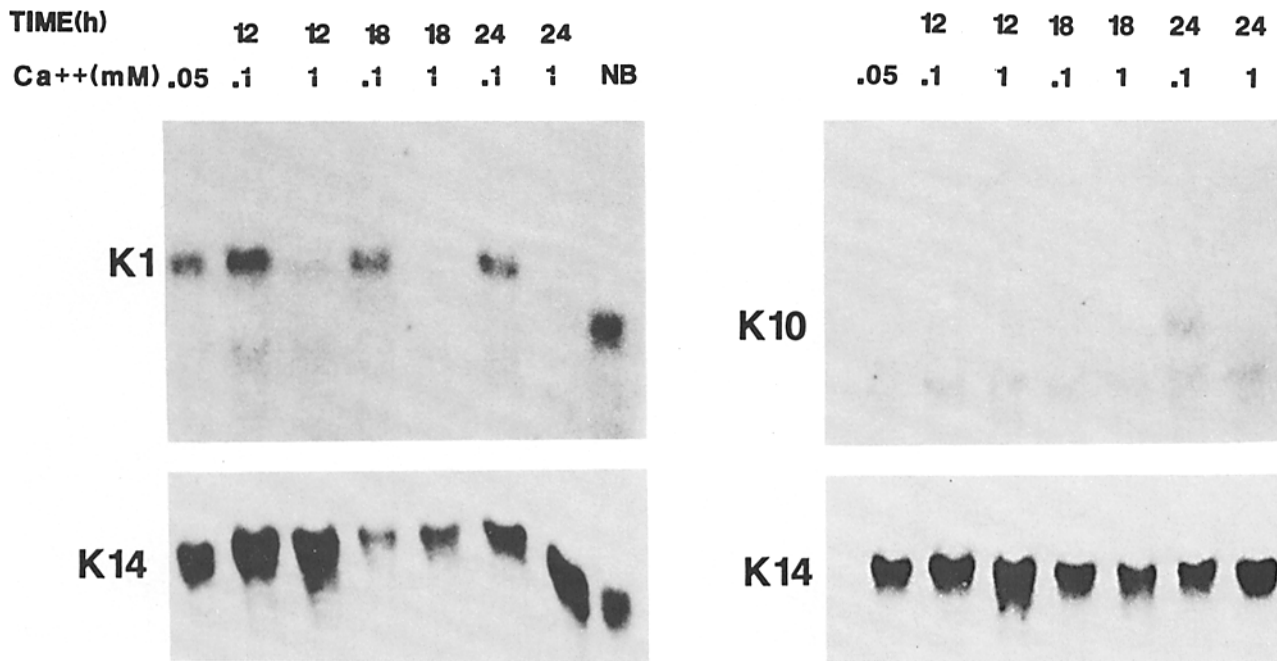


**Figure 5.** Detection of differentiation markers in cultured keratinocytes by indirect immunofluorescence. Primary cultures were prepared as in Fig. 1 and changed to 0.12 mM  $\text{Ca}^{2+}$  medium for 48 h. Ethanol-fixed dishes were reacted with monospecific guinea pig antiserum to K14 and monospecific rabbit antisera to K1, K10, CE, or filaggrin as described in Materials and Methods. Secondary antibodies were biotin-labeled goat anti-guinea pig IgG, which was visualized by streptavidin-Texas red, or FITC-labeled goat anti-rabbit IgG. Shown are double exposures of a single representative field for each antiserum. Yellow cells indicate coexpression of K14 and a suprabasal marker.

increase in K10 protein between 24 and 48 h. This suggests that optimal expression of K10 occurs between the 24- and 48-h time points studied. In contrast, CE (1.6-kb) and filaggrin (17-kb) transcripts, similarly dependent on 0.1 mM  $\text{Ca}^{2+}$  for optimal expression, became more abundant between 24 and 48 h. Furthermore, transcript levels were dependent on continued exposure to optimal  $\text{Ca}^{2+}$  since they decreased rather than increased if cells were changed to 0.5 mM  $\text{Ca}^{2+}$  after 24 h in 0.1 mM  $\text{Ca}^{2+}$ . The maximal expression of filaggrin and CE transcripts at a time later than that for K1 and K10 in response to the same signal of 0.1 mM

$\text{Ca}^{2+}$  was consistent with their later appearance during the differentiation program of the stratified epidermis in vivo. In the lower panels of Fig. 7, the blots were rehybridized with a K14 probe to assess gel-loading patterns.

To evaluate the possibility that  $\text{Ca}_o$  was regulating an inducible factor to control marker transcription, keratinocytes were exposed to 0.1 mM  $\text{Ca}^{2+}$  in the presence of cycloheximide for 24 h. Fig. 8 indicates that cycloheximide inhibited the increase in K1 message at permissive  $\text{Ca}_o$  while K14 transcripts were unaffected. These results suggest that a cycloheximide-sensitive inducible factor mediates the  $\text{Ca}_o$ -



**Figure 6.** Northern blot analysis for transcripts of K1 and K10 in cultured keratinocytes. Primary epidermal cells were plated at  $40 \times 10^6$  cells/150-mm culture dish and maintained in 0.05 mM  $\text{Ca}^{2+}$  medium for 6 d. Fresh medium with 0.05 mM  $\text{Ca}^{2+}$  was continued or changed to 0.1 or 1 mM for the indicated times. Two dishes per group were pooled, and RNA was extracted as described in Materials and Methods. Approximately 20  $\mu\text{g}$  total RNA/sample was separated by electrophoresis in 1.1% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized to nick-translated  $^{32}\text{P}$ -labeled specific DNA probes for K1 or K10. Filters were stripped by boiling and rehybridized to a specific K14 DNA probe shown in the lower panel. The 0.05-mM sample was harvested with the 24-h time points.

regulated response. Such a factor could enhance transcription or delay degradation of the relevant messages. The latter mechanism would require very rapid degradation of K1 message in the absence of an inducible factor to achieve the result shown in Fig. 8.

### Discussion

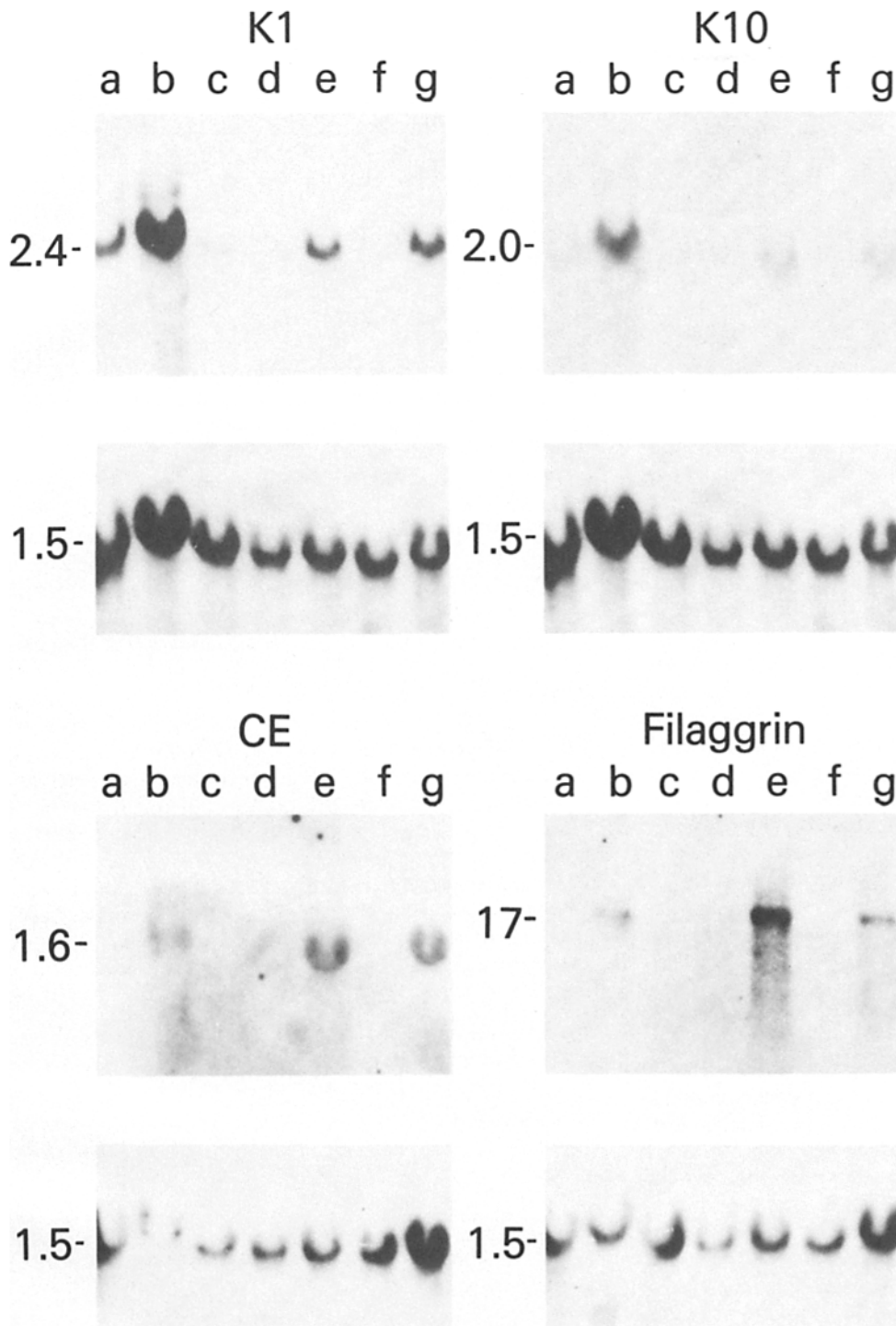
These studies demonstrate that specific markers of epidermal differentiation are expressed in cultured keratinocytes predominately in culture medium with a  $\text{Ca}^{2+}$  concentration restricted to 0.1–0.16 mM. Furthermore, at permissive  $\text{Ca}_0$ , marker expression is temporally coordinated. K1 and K10 are expressed before CE and filaggrin *in vitro* as *in vivo*. In some experiments, the  $\text{Ca}_0$  optimum for CE and filaggrin was slightly higher than for K1 and K10, but the differences were so small (0.02–0.1 mM) that they could not be firmly established as significant. Thus, keratinocytes may respond to a single  $\text{Ca}_0$  signal to turn on expression of both early and late differentiation markers. In some experiments, K1 expression was observed at a wider range of  $\text{Ca}_0$  than the other markers and coexpression was disassociated. This may indicate a more complex control of K1 expression. K1 is consistently found in a small number of basal epidermal cells *in vivo*, presumably under conditions where a basal cell  $\text{Ca}^{2+}$  environment is maintained. This report confirms that  $\text{Ca}_0$  regulates marker expression at the level of transcription and also indicates that a cycloheximide-sensitive  $\text{Ca}^{2+}$ -inducible factor may be involved.

Although K1, K10, CE, and filaggrin are expressed optimally within a limited range of  $\text{Ca}_0$ , cultured keratino-

cytes nevertheless morphologically differentiate and many cells with a squamous morphology will slough from the culture dish in all  $\text{Ca}_0 > 0.1$  mM (Hennings et al., 1980). The maturing cells constitute the majority of these high  $\text{Ca}^{2+}$  cultures and undergo many other changes that must not be tightly  $\text{Ca}_0$  restricted. These include loss of proliferative capacity, formation of desmosomes, expression of pemphigus antigen, formation of cross-linked cell envelopes, and other biochemical changes consistent with squame formation *in vivo* (Yuspa, 1983). Thus, programmed death of keratinocytes does not require expression of all of the components of squamous differentiation and multiple controlling processes are involved in achieving the fully differentiated phenotype. For example, cornified envelopes can be formed in the absence of expression of major cornified envelope precursors, although the structure of the envelope is abnormal (Nagae et al., 1987). Defective differentiation, of a sort where specific markers are abnormally expressed in the suprabasal layers, has been described in pathological conditions of the epidermis or after topical exposure to phorbol esters *in vivo* (Dover and Watt, 1987; Toftgard et al., 1985; Van Neste et al., 1988). These states may represent interruption of one of the several controlling elements in the differentiation process.

Even at the permissive  $\text{Ca}_0$ , we were not able to achieve marker expression in the majority of cells as determined by immunofluorescence analysis. Preliminary experiments suggest that a factor(s) in serum (other than vitamin A) reduces the number of expressing cells substantially. Wilke et al. (1988a,b) have suggested that terminal differentiation of human keratinocytes involves several steps. The first stage is a



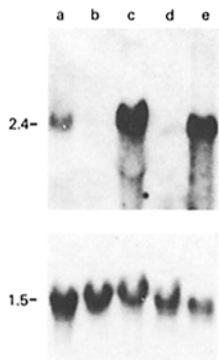


**Figure 7.** Northern blot analysis for transcripts of four suprabasal differentiation markers in cultured keratinocytes. Primary cultures were prepared as in Fig. 6 and exposed to varying  $\text{Ca}^{2+}$  concentrations: (lanes a-c) 0.05, 0.1, and 0.5 mM  $\text{Ca}^{2+}$ , respectively, for 24 h; (lanes d-f) 0.05, 0.1, and 0.5 mM  $\text{Ca}^{2+}$  respectively, for 48 h; (lane g) 0.1 mM  $\text{Ca}^{2+}$  for 24 h followed by 0.5 mM  $\text{Ca}^{2+}$  for 24 h. RNA was isolated and probed as described in Fig. 6. The K1 and K10 panels represent rehybridization of the same filter, while transcripts for CE protein and filaggrin were transferred to nitrocellulose from separate gels run in parallel. The filaggrin gel was exposed to 0.05 N NaOH to facilitate transfer of the very large size transcripts (Alwine et al., 1977). The bottom panel of each analysis is a rehybridization with a K14 probe.

reversible predifferentiation state characterized by cessation of proliferation. Cells in this state are then "receptive" to a true terminal differentiation signal such as  $\text{Ca}^{2+}$ . In the current studies we did not attempt to "prepare" cells for the proper  $\text{Ca}^{2+}$  signal so the majority of cells may not have been primed to respond. Future studies will be directed to define conditions (or other signals) which work in concert with  $\text{Ca}_0$  to induce marker expression in all cells.

These studies indicate that the temporal expression of specific markers in response to a common signal is inherently coordinated but can be disturbed if the proper signal

is not sustained. Thus, at 0.1-0.16 mM  $\text{Ca}^{2+}$ , K1 and then K10 and later CE and filaggrin are sequentially up-regulated at both the mRNA and protein levels. When the initiating signal is altered (e.g., changed from 0.1 to 0.5 mM  $\text{Ca}^{2+}$ ) before the time of optimal expression, less mRNA and protein accumulate. This phenomenon may explain altered expression of differentiation markers when transit time is accelerated in vivo. Assuming the  $\text{Ca}^{2+}$  gradient measured in the epidermis is functionally significant, then conditions which hasten the passage of a basal cell into the stratum corneum would interrupt the timing of  $\text{Ca}^{2+}$  signaling. Transit time



**Figure 8.** The influence of cycloheximide on the expression of the K1 transcript. Cultures were prepared as in Fig. 6 and exposed for 24 h to (lane *a*) 0.05 mM  $\text{Ca}^{2+}$ ; (lane *b*) 0.05 mM  $\text{Ca}^{2+}$  plus cycloheximide (2.5  $\mu\text{g}/\text{ml}$ ); (lane *c*) 0.1 mM  $\text{Ca}^{2+}$ ; (lane *d*) 0.1 mM  $\text{Ca}^{2+}$  plus cycloheximide; and (lane *e*) newborn epidermal poly A mRNA. The bottom panel is the blot rehybridized to a K14 probe.

and terminal differentiation are accelerated by phorbol ester treatment *in vivo*, and this acceleration markedly diminishes the expression of K1 and K10 (Toftgard et al., 1985). Conversely, conditions that provide a more orderly temporal program of differentiation might be expected to enhance marker expression. For example, cultured keratinocytes in monolayer growth generally stratify to a limited extent forming two to three cell layers in standard (1–2 mM)  $\text{Ca}^{2+}$  medium. However, when cultured on a floating collagen gel or in vitamin A-depleted culture medium, they stratify to a much greater extent. These methods are reported to be permissive for expression of suprabasal markers (Asselineau et al., 1985; Kopen et al., 1987), but only after sufficient time for the multiple cell layers to form. Excessive stratification itself could provide the correct spatial and temporal environment to allow for a more orderly, time-dependent expression of the differentiation-associated keratins. Stratification might also establish a  $\text{Ca}^{2+}$  gradient within the reformed tissue particularly since these methods commonly involve feeding cultures from below the tissue specimen.

It is likely that the specific  $\text{Ca}_o$  required to induce marker expression determines the establishment of a specific permissive intracellular  $\text{Ca}^{2+}$  which is the real mediator of the signal. Digital image analysis of Fura 2-loaded epidermal cells indicates that the intracellular  $\text{Ca}^{2+}$  concentration of normal keratinocytes increases in response to a change in the extracellular  $\text{Ca}^{2+}$  environment (Hennings et al., 1989). A similar phenomenon has been reported for bovine parathyroid cells (Nemeth and Scarpa, 1987). The intracellular change in keratinocytes is sustained during the course of the change in  $\text{Ca}_o$ , and the increment of change measured intracellularly is directly dependent on the increment of change in  $\text{Ca}_o$  (our unpublished observations). Furthermore, addition of ionomycin to 0.1 mM  $\text{Ca}^{2+}$  medium inhibits expression of all four markers studied and increases the intracellular  $\text{Ca}^{2+}$  above that observed in 0.1 mM  $\text{Ca}^{2+}$  medium alone (data not shown). These findings suggest that in response to a particular  $\text{Ca}_o$ , epidermal cells establish an intracellular  $\text{Ca}^{2+}$  milieu which is required to express differentiation markers.

Both extracellular and intracellular  $\text{Ca}^{2+}$  can influence the transcription or expression of differentiation-specific genes in other cell types. For example,  $\text{Ca}^{2+}$ , in conjunction with vitamin  $\text{D}_3$ , specifically increases the transcription of prolactin mRNA (Wark and Tashjian, 1983) in pituitary cells, and this is strictly dependent on the  $\text{Ca}_o$ . Parathyroid

hormone secretion is increased in 0.5 mM  $\text{Ca}^{2+}$  but decreased when bovine parathyroid cells are cultured in 1.25 or 2.5 mM  $\text{Ca}^{2+}$  (Silver et al., 1985).  $\text{Ca}^{2+}$ -calmodulin complex is required for the active transcription of the vitellogenin II gene in rooster nuclei while these factors were not required for transcription of lysozyme and ovalbumin (Jost et al., 1986). Thus, modification of intracellular  $\text{Ca}^{2+}$  is one mechanism involved in the selective expression of differentiation specific genes. Similarly, it has been proposed that certain rapidly transcribed genes associated with growth stimulation are regulated by a change in intracellular  $\text{Ca}^{2+}$  concentration (Berridge, 1987).

These studies provide a function for the  $\text{Ca}^{2+}$  gradient observed in epidermis *in vivo*. However, a mechanism whereby a  $\text{Ca}^{2+}$  gradient can be maintained is unclear. The epidermal basement membrane is composed of anionic proteins (Eady, 1988) that could have an affinity for  $\text{Ca}^{2+}$  as the serum filtrate crosses from the dermal capillaries. This would reduce the  $\text{Ca}_o$  in the basal cell compartment. In the more differentiated layers, dying cells could release stored intracellular  $\text{Ca}^{2+}$  and establish a retrograde gradient in the extracellular space. However, dynamic maintenance of such a gradient coupled with the narrow range required for marker responsiveness would suggest that controls other than concentration effects must be involved. In a model where ionic gradients are critical for differentiation control, conditions which disturb the gradient, such as wounding or basement membrane defects, might influence both differentiation and the control of proliferation. Since extracellular  $\text{Ca}^{2+}$  has now been shown to regulate the growth and differentiation of other lining epithelial cell types in addition to epidermal cells (Lechner et al., 1982; McGrath and Soule, 1984) the observations presented in this report may have implications beyond the control of differentiation in the skin. The establishment of ionic gradients across a basement membrane may be one general way in which epithelial differentiation is controlled.

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