

# Commitment to Differentiation and Expression of Early Differentiation Markers in Murine Keratinocytes In Vitro Are Regulated Independently of Extracellular Calcium Concentrations

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**Abstract.** In the epidermis, one of the earliest characterized events in keratinocyte differentiation is the coordinate induction of a pair of keratins specifically expressed in suprabasal cells, keratin 1 (K1) and keratin 10 (K10). Both in vivo and in vitro, extracellular calcium is necessary for several biochemical and structural changes during keratinocyte differentiation. However, it has been unclear if calcium serves as a differentiation signal in keratinocytes. In these studies, expression of suprabasal keratin mRNA and protein is used to test whether the initial differentiation of primary mouse keratinocytes in vitro is dependent on changes in the concentration of extracellular calcium.

K1 mRNA was expressed at low levels in cultures of keratinocytes growing on plastic in 0.05 mM calcium but in attached cells was not further induced by in-

creases in the concentration of extracellular calcium. Suspension of the keratinocytes into semi-solid medium induced a rapid and substantial increase in both expression of K1 mRNA and in the percentage of cells expressing suprabasal keratin proteins. The induction was unaffected by the concentration of calcium in the semi-solid medium and could not be enhanced by exposing attached cells to higher calcium before suspension. The induction of K1 mRNA could be inhibited by exposure of the keratinocytes to either EGF or fibronectin. These results suggest that commitment of mouse keratinocytes to terminal differentiation is independent of extracellular calcium and may be regulated primarily by extracellular factors other than calcium.

**T**ERMINAL differentiation of keratinocytes proceeds through a defined program of changes in gene expression, cellular architecture, and enzyme activation. Differentiation of the basal keratinocyte results in a permanent loss of growth potential and the subsequent sequential expression of differentiation markers; first expressed are the two suprabasal keratins K1 and K10 (Schweizer and Winter, 1983; Eichner et al., 1986) followed by other proteins, such as involucrin (Watt, 1983), filaggrin (Harding and Scott, 1983) and epidermal transglutaminase (Rice and Green, 1979), which are specific to later stages of differentiation. This pattern of expression in the maturing cell as it migrates upward from the basal layer of the skin allows the formation of an ordered, stratified epithelium. Both in vivo and in vitro studies suggest multiple events, including the inhibition of proliferation (Fuchs, 1990), and the loss of contact with the basement membrane (Adams and Watt, 1989), are necessary for keratinocytes to differentiate. However, it remains to be determined what specific cellular signals initiate the changes in gene expression in basal keratinocytes that commit them irreversibly to a program of differentiation.

Many recent studies have involved identifying cellular factors which regulate keratinocyte differentiation. Most studies have shown that changes in the concentration of extracellular calcium are required for at least some events in the pathway of terminal differentiation. In fact, there is evidence in vivo for a concentration gradient across the skin of extracellular calcium, which increases from a very low concentration in the basal layer to higher levels in the granular layer (Menon and Elias, 1991). Calcium clearly is essential for structural changes involving cellular architecture and adhesion, such as desmosome assembly and stratification (Watt et al., 1984; Duden and Franke, 1988; Hennings et al., 1980), and for activation of enzymes such as epidermal transglutaminase (Greenberg et al., 1991) that participate in late stages of differentiation.

It is less clear what role calcium has in regulating either growth or gene expression in keratinocytes. Although the culture of keratinocytes in low concentrations (less than 0.1 mM) of calcium enhances proliferation over differentiation, increasing extracellular calcium has both been found to inhibit and to stimulate cell growth depending on culture conditions (Hennings et al., 1980; Tsao et al., 1982; Wille et al., 1984; Shipley and Pittelkow, 1987; Watt et al., 1991). Similarly, the synthesis of differentiation markers, such as

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involucrin, and of desmosomal proteins integral to the stratification of the cells was observed under both low and high calcium concentrations (Watt et al., 1984; Duden and Franke, 1988; Fuchs and Green, 1981), and UEA 1 lectin binding was observed in rat keratinocytes maintained in low calcium (Ku and Bernstein, 1988). Increased concentrations of extracellular calcium did not induce expression of either suprabasal keratins or filaggrin (Fleckman et al., 1985; Kopan et al., 1987) in human keratinocytes, whereas in mouse keratinocytes expression of the suprabasal keratins (K1/K10) was induced by increasing calcium concentrations within a narrow range (Yuspa et al., 1989). Importantly, the variation in culture conditions between many of these studies suggests that the specific effect of calcium on both the growth and differentiation of keratinocytes is dependent on the physiological environment of the cells.

Determining the effect of calcium on differentiation *in vitro* is also complicated because both desmosome assembly and stratification require increases in extracellular calcium. As the expression of differentiation markers in keratinocytes grown *in vitro* occurs concurrently with stratification (Henning et al., 1980; Fuchs and Green, 1981), it is difficult to assess whether expression of these markers is influenced primarily by the increased calcium, or alternatively, by the stratification of the cells. Several studies using human cells have observed that expression of some differentiation markers can be induced without increasing extracellular calcium, either by treatment with phorbol esters (Watt, 1989; Dlugosz and Yuspa, 1993), or by loss of contact with the substratum (Green, 1977; Watt et al., 1988). Thus, it remains unclear whether calcium itself serves as a differentiation signal, or instead affects the expression of differentiation markers by interacting with other cellular factors or signal pathways.

To eliminate some of the variables present in previous studies, we used an optimized, defined medium, which allowed us to study growth and differentiation in the absence of serum factors that could affect both differentiation and the cellular response to calcium. We demonstrate that when this medium is used to culture primary mouse keratinocytes, cell suspension into semi-solid medium can efficiently induce expression of early markers of keratinocyte differentiation. This *in vitro* differentiation model, which possibly mimics the loss of basement membrane contact resulting from stratification is used to study the effects of calcium on keratinocyte differentiation independently of the stimulation of cell stratification. In this paper, we examine both direct and indirect effects of calcium; asking first whether the expression of early differentiation markers and commitment of cells to differentiation requires or is induced by increased extracellular calcium, and secondly, testing whether calcium inhibits cell growth and thereby indirectly stimulates differentiation (Glick et al., 1990).

We found that cell suspension induces differentiation independently of any change in extracellular calcium concentration. The induction of both keratin mRNA and protein was observed in 0.05 mM Ca<sup>++</sup>, and the induction of K1 mRNA was unaffected by increases in the extracellular calcium concentration in the semi-solid medium. Additionally, although higher extracellular calcium clearly promoted increased stratification in attached cells, it did not induce expression of K1 mRNA nor result in irreversible inhibition of cell growth. The results presented in this paper suggest that

calcium only indirectly affects the commitment of keratinocytes to differentiation and that common cellular factors other than calcium regulate differentiation in mouse and human keratinocytes.

## Materials and Methods

### Primary Cell Culture and Nuclear Labeling

Keratinocytes were isolated from the epidermis of newborn ICR mice essentially as described (Yuspa and Harris, 1974) except that mice were first killed with CO<sub>2</sub>. Isolated cells were plated into 60- or 100-mm plastic tissue culture dishes (Corning Glass Co., Corning, NY) at a density of 120,000 cells/cm. Primary keratinocytes were cultured in KBM medium (Clonetics Corp., San Diego, CA) (Shiple and Pittlekow, 1987), containing 0.05 mM calcium and supplemented with 0.5 µg/ml insulin (Clonetics Corp.), 0.05 µg/ml hydrocortisone (Sigma Chem. Co., St. Louis, MO; H0888), 0.1 ng/ml murine EGF (Collaborative Research, Bedford, MA, receptor grade), 7.5 µg/ml bovine pituitary extract (Clonetics Corp.), and 1% dialyzed bovine serum (GIBCO BRL, Gaithersburg, MD). This supplemented medium will subsequently be referred to in this paper as complete medium. Defined medium will refer to basal medium supplemented with insulin and hydrocortisone only. As indicated, 1% serum was added as a trypsin inhibitor and did not detectably affect differentiation in the primary mouse keratinocytes. Each experiment in these studies was repeated at least three times using independent isolations of primary cells.

Defined medium supplemented with 1.0% serum and containing 1.45% methylcellulose (Sigma Chem. Co.) (Green, 1977) was used as semi-solid medium. For subculture into semi-solid medium, keratinocytes were detached from plates by incubation at room temperature in a minimal volume of 0.1% Trypsin in HBSS, rinsed in defined medium containing 5% serum, and pelleted by centrifugation. Cells were resuspended in a small volume of defined medium and added to semi-solid medium at a density of 10<sup>6</sup> cells/ml and cultured in 15 × 100 mm polypropylene culture tubes (Falcon, Franklin Lakes, NJ). Fibronectin (Boehringer Mannheim Corp., Indianapolis, IN; Grade 1 from human serum) was solubilized in KBM at 4°C at 1 mg/ml before addition to semi-solid medium. Thymidine labeling and autoradiography was performed as previously described (Olson et al., 1993).

### Fixation and Immunohistochemical Staining of Cells

Keratinocytes grown on plastic dishes or coverslips were rinsed with TBS and fixed directly onto the plastic with 50% acetone/50% methanol for 2 min at -20°C, rehydrated in TBS at room temperature and used immediately for immunohistochemistry. Cells in semi-solid medium were diluted 10-fold with PBS (4°C) pelleted by centrifugation, rinsed 1 × with PBS and resuspended in a small volume of PBS. Cells were spun onto Superfrost Plus slides (Fisher, Pittsburgh, PA) with a cytocentrifuge, air dried 1-2 min to allow cells to affix to the slide, sample area was encircled with a PAP pen (Daido Sangyo Co.) and the slides were then rinsed in TBS. Slides were fixed in 50% methanol/50% acetone for 2 min at -20°C and stored for brief periods as required for additional time points. Cells were then rehydrated in cold TBS and used immediately for immunohistochemistry. Briefly, cells were first blocked in 10 mM Tris-Cl (pH7.4)/100 mM MgCl<sub>2</sub>/0.5% Tween/1% BSA/5% dialyzed FBS for 1 h at room temperature, and then rinsed with TBS and incubated overnight at room temperature with a monoclonal anti-cytokeratin K1/K10 antibody Ck 8.60 (Sigma Chem. Co., C7284) (Gigi-Leitner et al., 1986; Kopan et al., 1987) at a 1:100 dilution in TBS containing 1% Tween. After 3 × 15' rinses in TBS containing 1% Tween, the samples were incubated with a goat anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (Boehringer Mannheim Corp., 605260), diluted 1:1,000 in TBS containing 1% Tween. Samples were stained by standard techniques (Harlow and Lane, 1988) using BCIP/NBTZ as a substrate. No staining was detected in samples incubated with secondary antibody only, or with primary antibody in an undifferentiated mouse basal keratinocyte cell line (Balb/MK).

### Western Blotting

Cells were lysed directly in sample buffer (0.25 ml/10<sup>6</sup> cells) containing 2% SDS/60 mM Tris-HCl (pH 6.8)/10% glycerol and sonicated for 30 s (Tekmar Corp., Cincinnati, OH, microtip #0418). A small volume was removed and used for protein normalization using a BCA (bicinchoninic acid)

protein assay (Pierce, Rockford, IL). DTT was then added to a concentration of 100 mM and samples were heated to 100°C for 5 min. Sample volumes containing equal amounts of protein were analyzed by SDS-PAGE using an 8% separating gel and proteins were transferred to nitrocellulose (Trans-Blot membrane; Bio-Rad Labs., Hercules, CA) by electroblotting. The filter was blocked with a solution of 5% non-fat milk powder (Carnation) in TBS containing 0.2% Tween (TBST).<sup>1</sup> Suprabasal keratin proteins were detected by incubation with Ck 8.60 monoclonal antibody diluted 1:1,000 in TBST, followed by incubation with goat anti-mouse IgG secondary antibody conjugated to peroxidase (Boehringer Mannheim Corp., 605250) diluted 1:10,000 in TBST. Protein bands were detected using luminol as a chemiluminescent substrate (ECL, Amersham Corp., Arlington Heights, IL).

### RNA Isolation and Northern Analysis

Isolation of RNA and Northern analysis were performed as previously described (Drozdoff and Pledger, 1991). A variable amount of tRNA is coprecipitated by this RNA isolation procedure so that normalization of RNA samples by UV absorbance occasionally results in uneven loading of mRNA on gels; therefore, levels of a constitutively expressed stable mRNA (IB15-cyclophilin) which correlated well with ethidium bromide staining of 28s ribosomal RNA (data not shown), were used to normalize samples within gels. All Northern analyses were replicated at least three times using separate isolations of primary cells. Representative blots from each set of experiments are shown in figures. RNA probes for IB15, K1, and K14 were generated from the following plasmids: pBplB15; (pSP65; Promega Corp.) supplied with a 680 base pair BamHI-PstI insert from a cyclophilin cDNA clone (Danielson et al., 1988); pK1 and pK14; pGEM3 (Promega Corp.) supplied with short 3 min primarily noncoding inserts from cDNAs for K1 (suprabasal) and K14 (basal) keratins (Roop et al., 1983).

## Results

### Expression of Suprabasal Keratins in Attached Cells Is Not Induced by Extracellular Calcium

We first examined how the level of extracellular calcium affected differentiation in confluent cultures of keratinocytes attached to plastic dishes. Cells were treated with various

concentrations of calcium for 4 d and subsequently stained for expression of suprabasal keratins K1 and K10. As shown in Fig. 1 A, at low concentrations of calcium (0.05 mM) which inhibited stratification, K1/K10 expression was detectable by immunohistochemical staining in ~5% of the cells. The distribution of K1/K10 positive cells in the dishes was similar to the distribution of involucrin positive cells in human keratinocyte cultures maintained in low calcium (Watt and Green, 1982). Staining was restricted to rounded cells which were detaching from the monolayer, and we consistently found that a majority of the floating cells stained positively for suprabasal keratins (data not shown). This indicated that while differentiation could take place in low concentrations of calcium, with the inhibition of stratification, differentiating cells detached into the culture medium instead of forming layers.

After cells were cultured for 4 d in high calcium (1.0 mM) there was a noticeable increase in stratification accompanied by an approximately twofold increase in keratin positive cells to ~10% of total cell number (Fig. 1 B). Similarly, as in low calcium conditions, Fig. 1 B shows staining in some of the stratified cells and not in the underlying monolayer, suggesting that stratification alone was not sufficient to induce differentiation. Additionally, we observed that the number of floating cells markedly decreased in high calcium medium. This observation suggested that increased adhesion of differentiating cells rather than an increase in the rate of differentiation could account for the gradual increase in keratin positive cells when cultured in high calcium.

To further assess whether changes in extracellular calcium induced differentiation, we measured the level of K1 mRNA 24 h after cultures were switched from 0.05 mM to increasingly higher concentrations of calcium. As shown in Fig. 2 (lanes 1-4), expression of K1 mRNA was easily detectable in cells grown in 0.05 mM calcium, confirming the presence of differentiating cells. In contrast to some previous studies in which K1 expression was induced in primary mouse ker-

1. Abbreviation used in this paper: TBST, TBS containing 0.2% Tween.

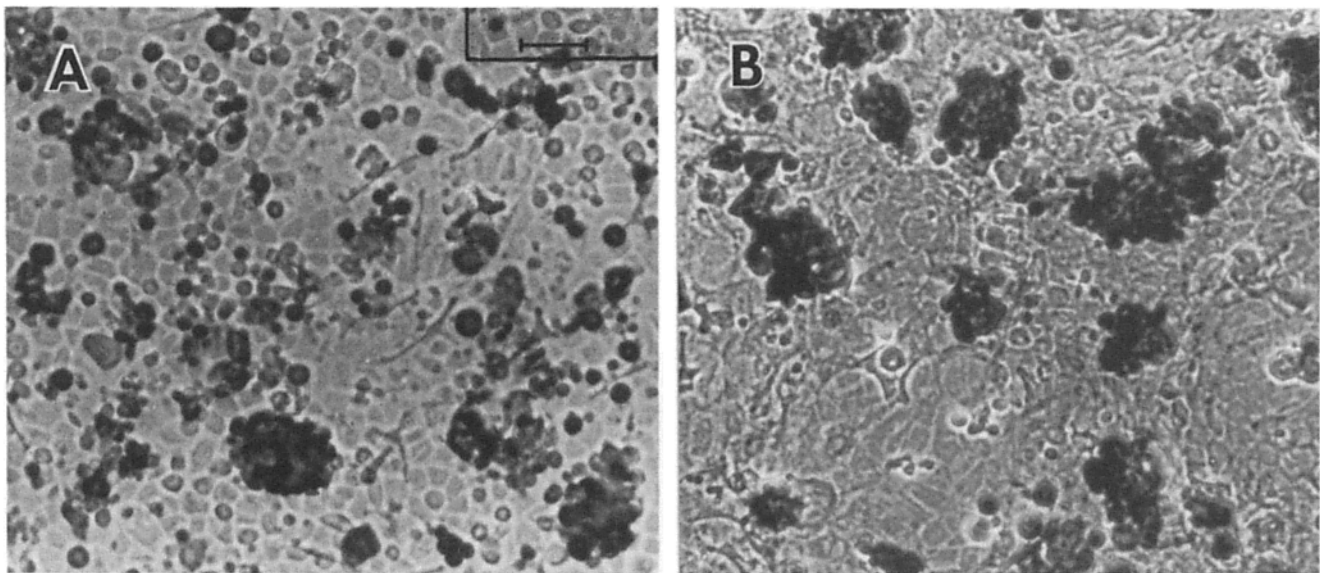
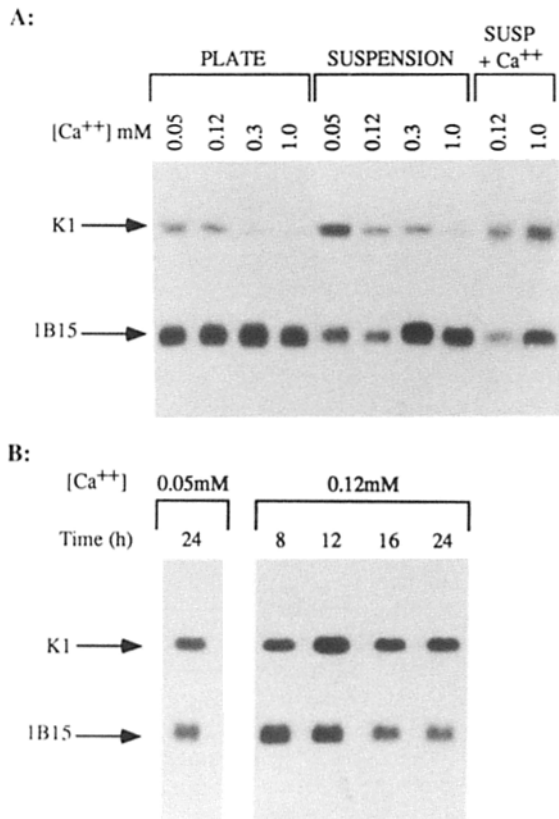


Figure 1. Immunohistochemistry of primary mouse keratinocyte cultures using Ck 8.60 monoclonal cytokeratin antibody. Cells were grown to confluence and incubated for 4 d in defined medium containing 1% serum and either 0.05 mM (A) or 1.0 mM (B) calcium. Bar, 40  $\mu$ m.



**Figure 2.** Effect of extracellular calcium on expression of K1 mRNA. Primary mouse keratinocytes were grown to confluence in complete medium containing 0.05 mM calcium. In *A*: Cells were then either: incubated for 24 h in defined medium containing the indicated concentration of calcium (*plate*); incubated for 24 h in defined medium containing the indicated concentration of calcium and then suspended into semi-solid medium containing 0.05 mM calcium and incubated for 16 h (*suspension*); incubated for 24 h in defined medium containing 0.05 mM calcium and then suspended into semi-solid medium containing either 0.12 or 1.0 mM calcium and incubated for 16 h (*SUSP + Ca<sup>2+</sup>*). In *B*: Cells were then incubated in defined medium containing either 0.05 or 0.12 mM calcium for the indicated times. Blots in *A* and *B* were hybridized with probes of different specific activities so that mRNA levels are not directly comparable between panels.

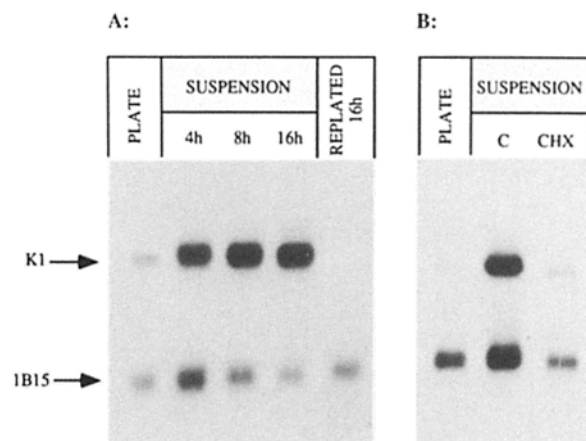
atinocytes by 0.10–0.15 mM calcium, increasing the calcium concentration to 0.12 mM had no apparent effect on the level of K1 mRNA. The level of K1 mRNA was similarly unchanged both at 12 and 16 h after increasing calcium to 0.12 mM (Fig. 2 *B*). Interestingly, increasing the calcium concentration to 0.3 mM and higher significantly decreased the expression of K1 mRNA, which would be consistent with the observation that keratin expression was detected only in some stratified cells grown in 1.0 mM calcium (Fig. 1).

### Induction of Suprabasal Keratins by Cell Suspension Is Independent of Extracellular Calcium

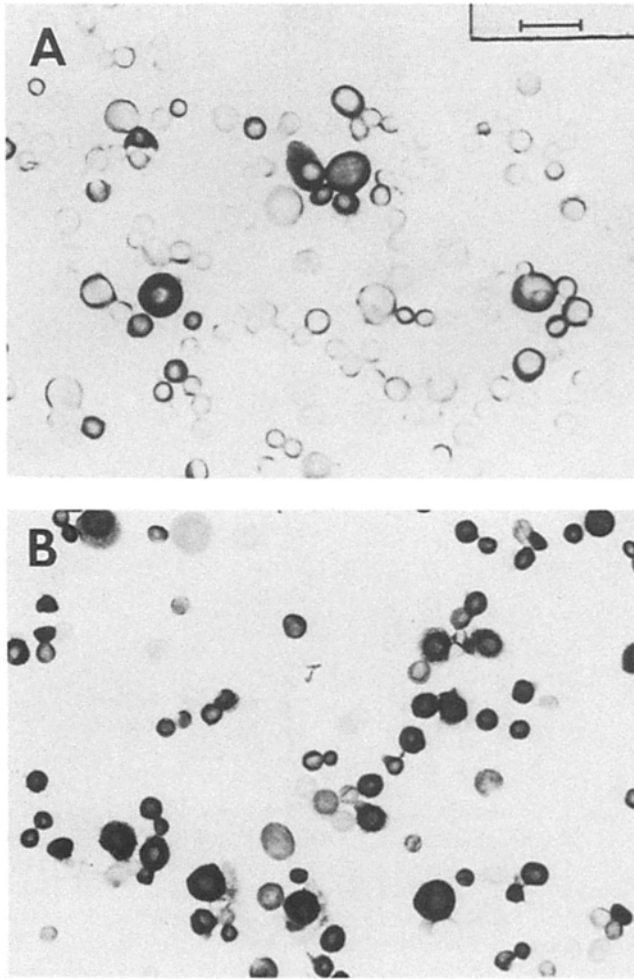
Suspension of primary human keratinocytes into semi-solid medium containing methyl cellulose has been well documented both to result in a dramatic loss of growth potential and to induce differentiation as indicated by the formation of cornified cell envelopes (Green, 1977). Recent studies dem-

onstrated that this treatment could also rapidly induce the expression of involucrin (Adams and Watt, 1989, 1990), but had not determined if this induction was dependent on specific extracellular calcium concentrations. To determine if incubation of primary mouse keratinocytes in semi-solid medium similarly induced expression of early differentiation markers and whether this induction was dependent on extracellular calcium, we first asked whether suspension of keratinocytes into low calcium semi-solid medium (containing 0.05 mM Ca<sup>2+</sup>) resulted in induction of K1 mRNA. Fig. 3 *A* clearly shows that without any increase in extracellular calcium, expression of K1 was induced severalfold. Increased expression of K1 mRNA was apparent at 4 h and peaked 12–16 h after cells were placed in suspension (see Fig. 7). The increase was inhibited by cycloheximide (Fig. 3 *B*), demonstrating that protein synthesis was necessary to induce expression of K1 mRNA. Fig. 7 also indicates that cell suspension caused a small decrease in the expression of mRNA for the basal cell specific keratin, K14 (relative to the level of IB15 mRNA).

To determine the percentage of cells induced to differentiate by cell suspension, we compared suprabasal keratin expression in individual cells before and after incubation in semi-solid medium. As indicated in Fig. 4 *A*, staining for K1/K10 was evident in a small percentage of the cells (~5%) before incubation in semi-solid medium but was restricted primarily to the larger cells in the population. The specific staining of larger cells in the culture is indicative of the progressive increase in cell size during keratinocyte differentiation (Sun and Green, 1976; Watt and Green, 1981). After incubation in suspension for 24 h, staining for K1/K10 was detected in a majority (50–80% among three experiments) of the cells (Fig. 4 *B*). The finding that most of the smaller cells expressed suprabasal keratins after suspension demon-



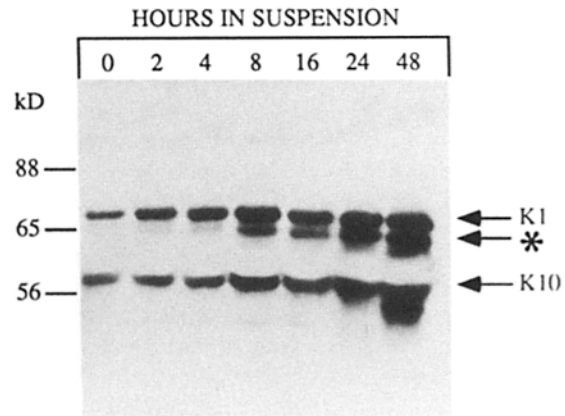
**Figure 3.** Incubation of primary mouse keratinocytes as a single cell suspension in semi-solid medium containing 0.05 mM calcium rapidly induces expression of K1 mRNA. Cells were grown to confluence in complete medium containing 0.05 mM calcium, and then incubated in defined medium (0.05 mM calcium) for 24 h. (*A*) K1 mRNA in attached cells before suspension (*plate*) is compared to K1 mRNA in cells which were then suspended into semi-solid medium (0.05 mM calcium) and incubated for 4–16 h as indicated (*suspension*). (*B*) Cells were incubated in semi-solid medium (0.05 mM calcium) for 16 h in the absence (*C*) or presence (*CHX*) of 1  $\mu$ g/ml cycloheximide.



**Figure 4.** Immunocytochemical staining with Ck 8.60 monoclonal antibody in individual cells after incubation in semi-solid medium. Keratinocytes were grown to confluence in complete medium (0.05 mM calcium), and then incubated in defined medium (0.05 mM calcium) for 1 d. Cells were then trypsinized from plates and immunohistochemistry performed as described, before (A) or after (B) 24 h of incubation in semi-solid medium containing 0.05 mM calcium. Bar, 40  $\mu$ m.

strated that cell suspension efficiently induced the differentiation of the majority of previously undifferentiated cells in the culture. Western blot analysis (Fig. 5) indicated that both suprabasal keratins K1 and K10 were induced after cell suspension, and that the total level of K1 protein increased rapidly after induction of K1 mRNA (Figs. 3, 5, and 7). These results clearly demonstrated that cell suspension could efficiently induce keratinocyte differentiation without requiring any change in extracellular calcium, and that the inability of calcium to induce differentiation in attached cells did not result from an inherent inability of the cells to differentiate *in vitro*.

To determine whether extracellular calcium and cell suspension had a synergistic effect on differentiation of primary mouse keratinocytes, the induction of K1 mRNA was compared in semi-solid medium containing different concentrations of extracellular calcium. Similar induction of K1 mRNA was observed in semi-solid medium with 0.05 mM



**Figure 5.** Western blot analysis demonstrating an increase in levels of suprabasal keratins K1 and K10 after incubation of primary mouse keratinocytes in suspension. Keratinocytes were grown to confluence in complete medium (0.05 mM calcium) and incubated for 1 d in defined medium (0.05 mM calcium). They were then resuspended into semi-solid medium (0.05 mM calcium) and incubated for the indicated periods of time. Ck 8.60 antibody was used for Western blot analysis as described. The relative positions on the blot of protein molecular weight markers are shown in kilodaltons on the left. The secondary protein below K1 (asterisk) cannot be definitively identified with the antibody used and could correspond either to a degradation product of K1 or to an additional suprabasal keratin (K2).

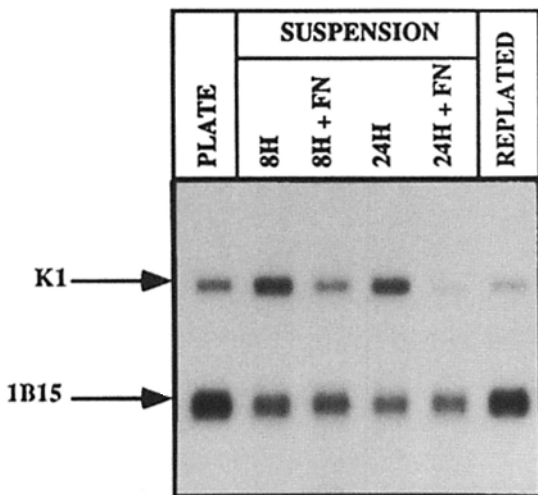
calcium (Fig. 2, lane 5) as with 0.12 or 1.0 mM calcium (Fig. 2, lanes 9–10), suggesting that the specific mechanism by which cell suspension committed cells to differentiation was independent of extracellular calcium. Secondly, we asked whether preexposure of attached cells to higher extracellular calcium enhanced differentiation when the cells were subsequently cultured in suspension. We found that cell suspension resulted in similar induction of K1 mRNA in cells preexposed to 0.05 mM calcium and to 0.12 mM calcium (Fig. 2, lanes 5–6), which was the concentration previously reported as optimal for expression of K1 mRNA (Yuspa et al., 1989). Interestingly, preincubation of the keratinocytes before cell suspension, in medium containing 0.3 and 1.0 mM calcium resulted in a decrease in the subsequent induction of K1 mRNA (Fig. 2, lanes 7–8). This result is similar to how higher extracellular calcium decreased K1 mRNA levels in attached cells. From these experiments alone it is not possible to evaluate whether increased extracellular calcium actually blocked differentiation, or instead specifically inhibited keratin expression in differentiating cells.

#### ***EGF and Fibronectin Are Regulators of Differentiation in Mouse Keratinocytes***

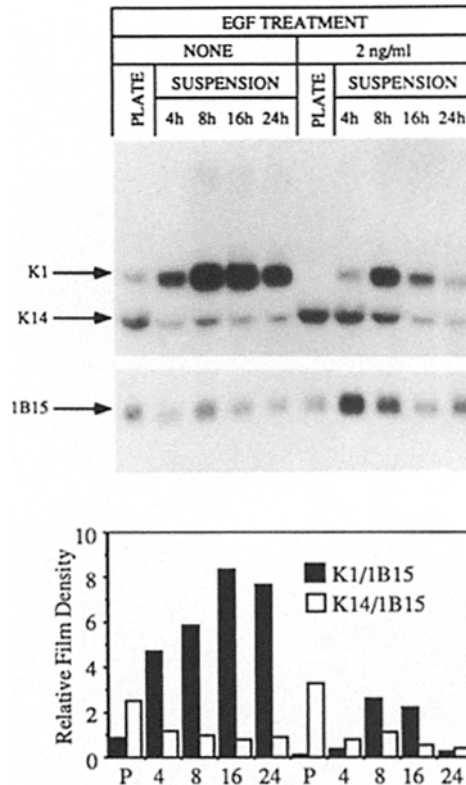
As different studies have found contrasting effects of calcium on differentiation between primary mouse and primary human keratinocytes, it has been argued that mouse and human cells differ significantly in their responsiveness to calcium and that differentiation in human cells may be regulated primarily by other factors (Kopan et al., 1987; Fuchs, 1990). However, the evidence in this study indicates that calcium does not directly induce differentiation in mouse keratinocytes and therefore, suggests that common factors regulate

differentiation in mouse and human keratinocytes. This premise was tested by examining whether two factors that had been previously shown to affect differentiation in human keratinocytes, fibronectin, and EGF, similarly influenced differentiation in mouse keratinocytes. In human keratinocytes, previous studies had shown that expression of involucrin induced by cell suspension was inhibited by the addition of fibronectin to the semi-solid medium (Adams and Watt, 1989). Confluent primary mouse keratinocytes were trypsinized and either replated onto plastic dishes or suspended into semi-solid medium in the presence or absence of 100  $\mu\text{g}/\text{ml}$  of fibronectin. Fig. 6 shows that fibronectin clearly inhibited the induction of K1 mRNA after cell suspension. Exposure to fibronectin had little effect on K1 expression in attached cells and inhibited differentiation only if directly added to the semi-solid medium (data not shown).

Evidence from several studies implies that EGF functions as an inhibitory factor for differentiation in human keratinocytes (Turksen et al., 1991; Marchese et al., 1990; Wille et al., 1984). To determine if EGF had a similar effect on differentiation in mouse keratinocytes, we examined the effect of EGF on induction of K1 mRNA after cell suspension. Interestingly, in contrast to fibronectin treatment, EGF had no effect on the induction of K1 when cells were exposed to EGF after they were suspended into semi-solid medium (data not shown). In attached cells however, a 24-h exposure to EGF inhibited the expression of K1 mRNA (Fig. 7, lane 6). Inhibition was evident at 6–8 h after EGF treatment and maximal after 24 h of exposure (data not shown). The EGF treatment also inhibited the subsequent induction of K1 mRNA when the cells were suspended into semi-solid medium (Fig. 7, lanes 7–10). Although maximal levels of K1 mRNA were inhibited, differentiation did not appear to be delayed since the induction of K1 after cell suspension in both EGF pretreated cells (lanes 7–10) and in control cells



**Figure 6.** Addition of fibronectin to semi-solid medium inhibits the induction of K1 mRNA after cell suspension. Keratinocytes were grown to confluence and incubated in defined medium for 1 d. The cells were then either suspended into semi-solid medium and incubated for 8 or 24 h in presence (+FN) or absence (-FN) of 100  $\mu\text{g}/\text{ml}$  of fibronectin (*suspension*), or replated on plastic dishes in defined medium and incubated for 24 h (*replated*).



**Figure 7.** Exposure to EGF before cell suspension inhibits induction of K1 mRNA. Keratinocytes were grown to confluence and incubated for 1 d in defined medium. The cells were then further incubated in the defined medium or in defined medium containing 2 ng/ml EGF for 24 h, as indicated. Cells were either harvested for RNA (*plate*) or resuspended and incubated in semi-solid medium for the indicated times (*suspension*). The relative intensity of bands representing K1 and K14 mRNA, normalized to IB15, was quantitated by laser densitometry and is plotted below the blot.

(lanes 2–5), followed a similar time course. This finding, that two factors shown to affect human keratinocyte differentiation have similar effects on differentiation in mouse keratinocytes, supports the notion that differentiation in both species is regulated by common mechanisms. As discussed further, they also suggest that both growth and extracellular matrix factors may serve as primary signals for keratinocyte differentiation.

### **Both Growth Arrest and Stratification Are Prerequisites for Differentiation in Attached Cells**

In both mouse and human keratinocytes, stratification of cells is substantially increased in high calcium (Hennings et al., 1980; Boyce and Ham, 1983; and see Fig. 1). In attached cells, as shown in Fig. 1, stratification appears to be necessary but not sufficient for the expression of early differentiation markers (because expression of suprabasal keratins is detected in only a minority of stratified cells), suggesting that differentiation is promoted by stratification but requires additional steps. One important step may be inhibition of cell growth. Both studies *in vitro* and the close coupling of growth arrest with differentiation in keratinocytes *in vivo*



have suggested that growth inhibition is an important prerequisite for differentiation (Wilke et al., 1988; Adams and Watt, 1989; Fuchs, 1990). In mouse keratinocytes, in culture conditions in which high calcium has been found to induce differentiation, there is a permanent loss of cell proliferation (Hennings et al., 1980; Yuspa et al., 1989). In contrast, several studies have found that growth of human keratinocytes is unaffected by the levels of extracellular calcium (Wille et al., 1984; Shipley and Pittelkow, 1987; Pillai et al., 1990), or that increasing extracellular calcium has a mitogenic effect (Watt et al., 1991). These observations suggest that the specific effect of high calcium on proliferation under different culture conditions may in part account for the differing effects that calcium has been observed to have on differentiation among mouse and human keratinocytes. Representative results from experiments performed to test if the growth of the primary mouse keratinocytes was inhibited in high calcium under the same conditions we had used to examine differentiation are illustrated in Fig. 8. Cells were grown to confluence in complete medium and then incubated for 3 d in defined medium containing either 0.05 mM (A and B) or 1.0 mM (C and D) calcium. Cells were then incubated for 24 h in the presence of tritiated thymidine with fresh medium containing either 0.05 mM (A and C) or 1.0 mM (B and D) calcium. In control cells, grown in 0.05 mM calcium and refed with 0.05 mM calcium (A), growth was restimulated in ~90% of the cells. Fig. 8 B shows that refeeding instead with high calcium medium similarly stimulated growth in ~90% of the cells, demonstrating importantly that during the time that calcium was observed to induce differentiation markers in other studies (Yuspa et al., 1989), there was no detectable growth inhibition by calcium, as well as no detectable increase in expression of K1 mRNA (Fig. 2).

Interestingly, as also seen in Fig. 8 B, growth was not markedly inhibited in areas of the dish which were beginning to stratify (arrow), demonstrating that stratification occurred before any obvious inhibition of growth. A lower percentage of cells were labeled when cultures first incubated in high calcium were refed with high calcium medium (8 D), compared to cultures incubated in low calcium and refed with high calcium medium (8 B). Growth inhibition is most evident in areas showing substantial stratification (arrows), previously shown to contain differentiating cells (Fig. 1), however stratification would prevent detection of labeled cells in the underlying basal layer. Direct quantitation of incorporated counts (Fig. 8, legend) indicated that most cells continued to proliferate in high calcium.

To test whether the growth inhibition was dependent on stratification, cultures incubated in high calcium were refed with low calcium medium. As the adherence of stratified cells is dependent on high calcium, the switch to low calcium results in the rapid detachment of much of the stratified layer, leaving a largely unstratified monolayer on the dish. Interestingly, as shown in Fig. 8 C, a very similar percentage of these remaining cells were labeled as in cells first incubated in low calcium and refed in low calcium shown in Fig. 8 A. These results demonstrate that in our culture conditions, exposure of the keratinocytes to high extracellular calcium does not by itself irreversibly arrest growth. Rather, they suggest that growth inhibition is dependent on stratification, as most of the cells in the underlying monolayer could be restimulated to grow. Since these cells retained the capacity for prolifera-

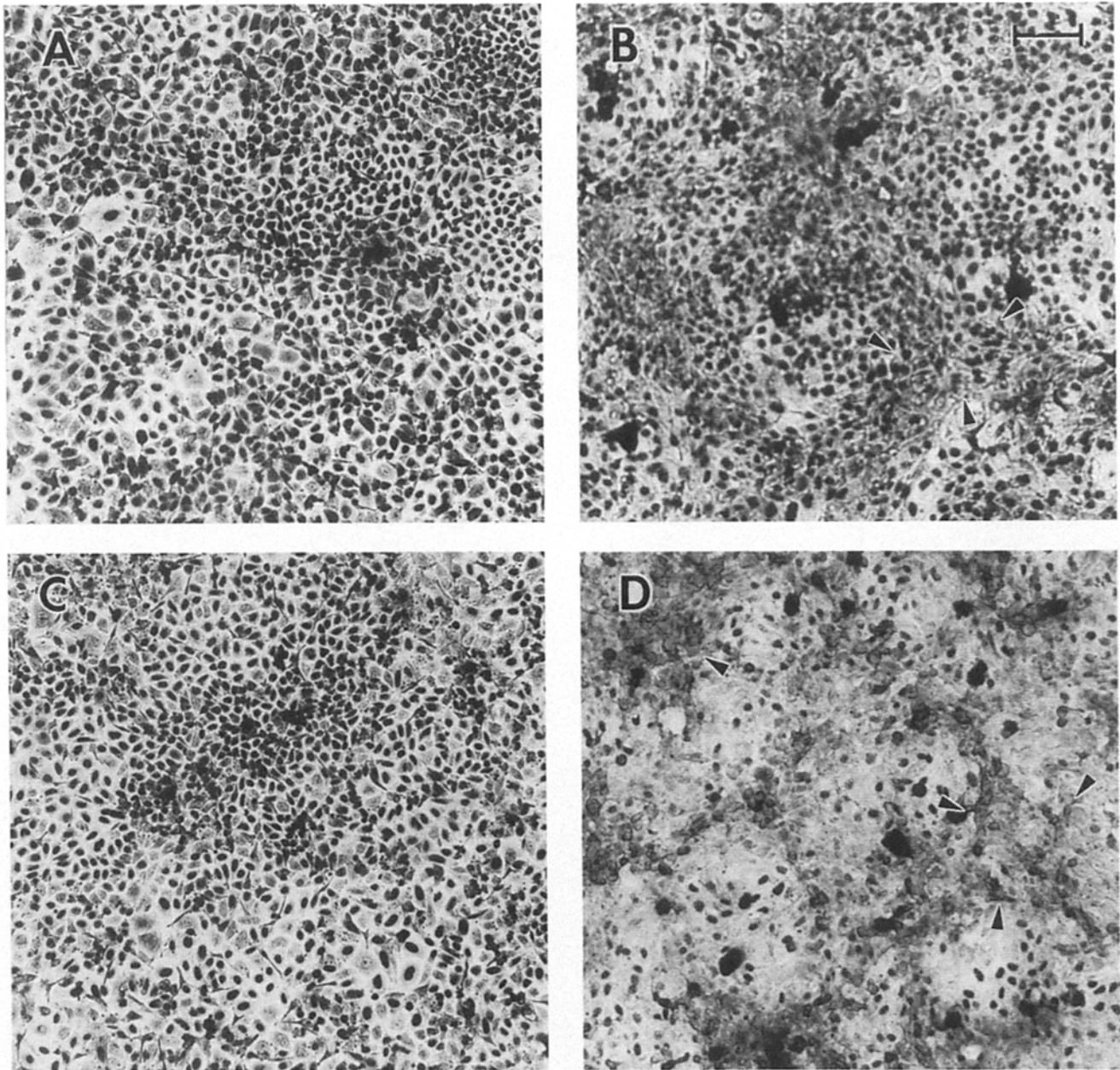
tion, these results also indicated that the underlying monolayer of unstratified cells remained undifferentiated after extended exposure to high calcium, supporting our hypothesis that calcium only indirectly affects terminal differentiation in vitro by promoting stratification of the cells.

## Discussion

Growth and differentiation of keratinocytes are coordinately regulated by a complex set of signals involving growth factors, hormones, extracellular matrix proteins, and calcium ions (Fuchs, 1990; Aneskevich et al., 1992; Watt, 1989). It is evident that by optimizing these parameters, both growth and differentiation can be balanced so that many features of normal epithelium may be reproduced in skin-equivalent in vitro culture systems (Choi and Fuchs, 1990). Nonetheless, because of the intricate interplay between many of these extra- and intracellular signals, it has been difficult to determine the specific roles that any individual factor has in regulating either proliferation or differentiation. A central issue is whether calcium functions as a primary signal in initiating terminal differentiation. While the proportion of differentiated cells attached to solid substrates is apparently decreased in low calcium, this may be explained by the inability of these differentiated cells to remain attached, and thus to stratify.

One problem, especially with in vitro systems in evaluating the effect of calcium and other extracellular factors on keratinocyte differentiation, has been what to use as a marker for differentiation. Cornified envelope formation reflects a late stage in the process and is dependent on several additional steps after cells commit to differentiation, including the expression of several protein precursors, and the expression and activation of epidermal transglutaminase (Watt, 1983; Rice et al., 1988). As these steps are either blocked by or dependent on several factors such as retinoic acid, hormones, serum factors, and especially calcium (Fuchs, 1990), the commitment of cells to terminal differentiation is not necessarily reflected in an increase in cornified envelope formation. Additionally, increased cornified envelope formation in culture can result from stimulation of a number of these later steps in differentiation without an increase in the rate of differentiation in the basal stem cell population. The pattern of expression of the suprabasal keratins K1/K10 in the skin suggests that they are among the earliest markers for keratinocyte differentiation (Eichner et al., 1984), and that K1/K10 expression would most clearly correlate in vitro with when cells had been induced to differentiate. Thus, in these studies, we used K1/K10 expression as a marker for differentiation.

In these studies, we show that cell suspension functions as an extremely potent technique for synchronously inducing differentiation in primary mouse keratinocytes. Although the structural changes occurring later in keratinocyte differentiation would not be reproduced in suspension-induced differentiation, it provides a good model for studying the initial cellular and molecular events taking place during differentiation. Our results clearly show that the induction of expression of early differentiation markers does not require changes in extracellular calcium. In contrast to earlier interpretations that calcium acts as a primary signal for differentiation in mouse keratinocytes, our results suggest



**Figure 8.** Effect of calcium on growth restimulation in confluent cultures of primary mouse keratinocytes. Confluent keratinocytes in 24 well plates were incubated in defined medium containing 1% serum and 0.05 mM (A and B) or 1.0 mM calcium (C and D) for 4 d (refed on day 2). The cells were then incubated for 24 h in fresh defined medium containing 0.05 mM (A and C) or 1.0 mM calcium (B and D), and 1  $\mu$ M tritiated thymidine. After labeling, the cells were fixed with methanol and processed for autoradiography. Bar, 160  $\mu$ m. In 8 D, stratified layers were too thick to allow complete exposure of the emulsion. Incorporated thymidine was measured in duplicate parallel wells by solubilizing the cells in 0.1 N NaOH/1% SDS and counting in a scintillation counter. Average incorporation compared to control cultures (473,000 dpm/well) (8 A), was 96% (B), 93% (C), and 58% (D).

that calcium indirectly promotes differentiation *in vitro* primarily through its effects on cell stratification. How differentiation is promoted by stratification is not clear, but as discussed below, both extracellular matrix factors as well as growth factors may serve as differentiation signals, and it is reasonable to speculate that stratification may affect both growth- and extracellular matrix-factor binding to the cells.

The effects that EGF and fibronectin have on suspension-induced differentiation give some clues as to the types of cel-

lular signals which are important in regulating differentiation, and to when commitment to differentiation occurs. Previous studies tested the hypothesis that cell suspension promoted keratinocyte differentiation through a loss of extracellular matrix contact by determining if fibronectin could inhibit suspension-induced increase in involucrin expression (Adams and Watt, 1989). Our finding that fibronectin added to the semi-solid medium inhibited keratin expression in mouse keratinocytes and previous observations that fibronectin in-



hibited involucrin expression in human keratinocytes support the conclusion that cellular binding of fibronectin blocks keratinocyte differentiation and provide additional evidence that extracellular matrix can serve as a primary signal in keratinocyte differentiation.

In this study, increased expression of suprabasal keratin mRNA was detected 4–8 h after cells were suspended into semi-solid medium. Both this and earlier studies (Adams and Watt, 1989) where it was found necessary to add fibronectin no later than 4 h after suspension of human keratinocytes in order to maximally inhibit induction of involucrin, would suggest commitment to differentiation in keratinocytes takes place as soon as 4 h after cell suspension. Interestingly, the earlier maximal induction in suspension of K1 we observed to that of involucrin (12–16 h compared to 24 h) may reflect the earlier expression of K1 *in vivo*.

As there is substantial evidence that EGF is a mitogen for keratinocytes, it is not surprising that high levels of EGF have been observed in previous studies to inhibit keratinocyte differentiation in culture (Wille et al., 1984). However, as with other agents influencing differentiation, the *in vitro* effect of EGF has been evaluated in terms of late markers for differentiation such as cornified envelope formation. Our data demonstrate that EGF treatment has a rapid effect on levels of K1 mRNA and is important in showing that EGF can mediate early, as well as late events in differentiation. The reduced induction of K1 mRNA in cells pretreated with EGF before suspension suggests that EGF may block cells from responding to differentiation signals. Interestingly, results from preliminary experiments, in which attached cells were treated with EGF in the presence and absence of actinomycin D, suggest that the inhibition of K1 mRNA is mediated in part through a reduction in message stability. This indicates that EGF may also act to modify expression of differentiation markers in cells once they have committed to differentiate. The inability of EGF to block differentiation when added to the semi-solid medium is intriguing and may suggest that keratinocytes are unable to respond to EGF when placed into suspension. We have previously observed that suspension of fibroblasts causes a dramatic reduction in the ability of both EGF and PDGF to bind to the cells (Drozdoﬀ, V., and W. J. Pledger, unpublished observations) and are investigating further whether a similar effect occurs for EGF binding in suspended keratinocytes. The loss of growth factor binding may provide an additional signal for differentiation in suspended cells.

Most importantly, the data presented in this paper suggests that induction of keratinocyte differentiation *in vitro* is not directly regulated by extracellular calcium. Previous studies have not resolved the important issue of whether calcium regulates gene expression during differentiation, and thus has a direct effect on differentiation. As discussed previously, most studies of human keratinocytes have not found convincing evidence for this but there is conflicting evidence in studies using mouse cells. The best evidence is provided in one series of studies, in which induction of mRNA for suprabasal keratins was observed when extracellular calcium was raised from 0.05 mM to between 0.10 mM and 0.15 mM (Yuspa et al., 1989). However, we found K1 mRNA expressed in cultures at concentrations of calcium between 0.05 and 0.15 mM while Yuspa et al. (1989), did not detect appreciable levels under 0.07 mM concentrations of calcium.

These differences may reflect the different culture conditions used between the two studies. In previous studies, keratinocytes were grown in a basal medium requiring a high concentration of serum. The presence of factors in the serum inhibitory for differentiation (Yuspa et al., 1989; Kopan et al., 1987) may have resulted in culture conditions less permissive for both differentiation and keratin expression. We find that in attached cells both that K1 mRNA is expressed at substantial levels in 0.05 mM calcium and is not further induced by increasing extracellular calcium to concentrations of 0.1 mM and higher affecting both cell morphology and stratification. In contrast to earlier interpretations, this directly argues that early marker expression is not restricted by low concentrations of extracellular calcium.

The conclusion that the effect of calcium on keratinocyte differentiation is indirect is supported by additional data. First, the ability of cell suspension to induce keratin expression in 0.05 mM calcium (Figs. 3–5) clearly shows that other factors can act as primary signals for differentiation without any change in the concentration of extracellular calcium. Second, in attached keratinocytes grown in high calcium, suprabasal keratins are detected only in stratified cells (Fig. 1). While it may be argued that stratification itself is a consequence of differentiation, the observation that cell growth is not markedly inhibited in stratifying cells (Fig. 8B) supports the opposite interpretation that growth arrest and differentiation occurs as a consequence of stratification. Furthermore, even after extensive stratification of the cells has occurred in high calcium, both this and previous studies (Roop et al., 1987; Yuspa et al., 1989) find that early differentiation markers are expressed in only a small percentage of the stratified cells. Additionally, although transcripts for K14 disappear rapidly in the first layer of suprabasal cells *in vivo* (Roop et al., 1988), previous studies found that *in vitro* the level did not change in response to changes in extracellular calcium (Yuspa et al., 1989). On the other hand, our results demonstrate that cell suspension induces both a substantial increase in K1 mRNA and early marker expression in a majority of the suspended cells, and furthermore, the induction of K1 mRNA is accompanied by a simultaneous decrease in mRNA for the basal keratin K14 relative to 1B15 (Fig. 4).

The observation that an increase in extracellular calcium induces expression of differentiation markers in keratinocyte monolayers attached to plastic in some culture conditions but not in others, as demonstrated here, may reflect the growth state of the cells in higher calcium. We propose that both growth arrest, as previously suggested (Wilke et al., 1988; Pittelkow et al., 1986; Watt et al., 1988), and additionally, stratification as implied by our data, are prerequisites for differentiation in attached cells. Previous observations have shown when human keratinocytes are maintained at high densities without refeeding, the cells enter a reversible growth arrest state due to depletion of critical amino acids in the medium (Pittelkow et al., 1986). Medium composition and culture conditions may thus be expected to easily affect the growth state of keratinocytes *in vitro*. In conditions favoring cell proliferation, the cells may be unable to respond to differentiation signals. For example, in rat keratinocytes, the amount of UEA 1 lectin binding was found to be inversely related to the fraction of proliferating cells (Ku and Bernstein, 1988). This would explain why differentiation would be delayed after stratification under our conditions in which

underlying cells remain in a proliferative state even after extended exposure to high calcium and arrest growth only after stratifying (Fig. 8 C). Differentiation would be induced more quickly under other conditions where growth arrest in unstratified cells would "prime" cells to respond to stratification. The rapid loss of proliferative potential in human keratinocytes after cell suspension (Adams and Watt, 1989; Pittelkow et al., 1986; Watt et al., 1988) suggests the effectiveness of cell suspension in inducing differentiation may result from rapid growth arrest combined with the loss of extracellular matrix binding.

Both in these and previous studies, expression of K1 mRNA in keratinocytes attached to plates was lowest in high extracellular calcium. In our studies, pretreatment with high calcium also inhibited the subsequent expression of K1 mRNA after cell suspension. There is substantial evidence that protein kinase C mediated signal transduction plays an important role in keratinocyte differentiation, and that extracellular calcium in part regulates activation of this pathway (Jaken and Yuspa, 1988; Toftgard et al., 1985; Dlugosz and Yuspa, 1993). Interestingly, several studies have observed that TPA induces the expression of late markers of differentiation but inhibits expression of the suprabasal keratins K1 and K10 (Roop et al., 1987; Molloy and Laskin, 1987). The inhibition of keratin expression by calcium would be consistent with the interpretation that a calcium-mediated activation of protein kinase C is involved in repression of the expression of early differentiation markers that occurs as keratinocytes move from the spinous to granular layer in the skin. Recently described calcium sensitive elements in the 3' non-coding region of the K1 gene may also be involved in mediating this response (Huff et al., 1983). While extracellular factors other than calcium may induce keratinocytes to differentiate, these observations indicate calcium may be important in regulation of gene expression later in differentiation.

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