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The Calcium Signal for BALB/MK Keratinocyte Terminal Differentiation Counteracts Epidermal Growth Factor (EGF) Very Early in the EGF-Induced Proliferative Pathway

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BALB/MK mouse epidermal keratinocytes require epidermal growth factor (EGF) for proliferation and terminally differentiate in response to high calcium concentrations. We show that EGF is an extremely potent mitogen, causing BALB/MK cultures to enter the cell cycle in a synchronous manner associated with a greater than 100-fold increase in DNA synthesis. Analysis of the expression of proto-oncogenes which have been reported to be activated during the cascade of events following growth factor stimulation of fibroblasts or lymphoid cells revealed a very rapid but transient 100-fold increase in c-*fos* RNA but little or no effect on the other proto-oncogenes analyzed. Exposure of EGF-synchronized BALB/MK cells to high levels of calcium was associated with a striking decrease in the early burst of c-*fos* RNA as well as the subsequent peak of cell DNA synthesis. Since the inhibitory effect of high calcium on c-*fos* RNA expression was measurable within 30 min, our studies imply that the EGF proliferative and calcium differentiation signals must interact very early in the pathway of EGF-induced proliferation. Our results also establish that c-*fos* RNA modulation is an importent early marker of cell proliferation in epithelial as well as mesenchymal cells.

Investigations of the intracellular pathways by which growth factors activate normal cellular proliferation have increased with emergence of evidence that genes coding for growth factors or their receptors can be altered so as to become involved in the malignant process. Conserved cellular genes, termed proto-oncogenes, transduced as the oncogenes of transforming retroviruses, have been shown in one case to encode a normal growth factor (10, 45) and in two more recent instances to encode the activated forms of different growth factor receptors (12, 37, 43). Moreover, the products of several other proto-oncogenes appear to be involved in pathways by which growth factors and mitogens stimulate normal cellular growth (4–7, 20, 27, 31, 33, 42).

Efforts to dissect normal growth regulation of epithelial cells have been aided in recent years by the development of tissue culture systems for propagation of such cells. One such system involves cloned BALB/MK cell lines derived from BALB/c mouse keratinocytes (46). These lines absolutely require epidermal growth factor (EGF) for their proliferation and terminally differentiate in response to an extracellular Ca^{2+} concentration greater than 1.0 mM (46, 47). The high Ca^{2+} differentiation signal is able to overcome the EGF proliferative stimulus. Recent evidence indicates that a number of retroviral oncogenes can both supplant the EGF requirement and block the Ca^{2+}-induced terminal differentiation pathway of BALB/MK cells (47). All of these findings have suggested that there may be interactions between the proliferation and differentiation pathways induced by these two signals.

In the present studies, we sought direct evidence of interaction between the pathways by which EGF and high Ca^{2+} stimulate normal BALB/MK cell proliferation and differentiation, respectively. We compared the effects of these stimuli on BALB/MK cellular DNA synthesis and on the expression of different proto-oncogenes. In combination with genetic evidence derived from analysis of spontaneous BALB/MK mutants, our findings reveal that these signals interact at an early point in the normal pathway of BALB/MK proliferation associated with the rapid modulation of c-*fos* mRNA levels.

MATERIALS AND METHODS

Cells and cell culture conditions. BALB/MK cells (46) were grown in minimal essential medium (MEM) containing a calcium concentration of 0.05 mM supplemented with 10% dialyzed fetal calf serum (FCS) (Gibco Laboratories) and EGF (Collaborative Research) (4 ng/ml). A clonal line, BALB/MK-2, was used in all of the experiments. When indicated, $CaCl_2$ was added at a final concentration of 1.5 mM to induce terminal differentiation. Mutant cell lines, BALB/MK EGF^I and BALB/MK Ca^r, were obtained by selecting BALB/MK-2 in medium lacking EGF or containing 1.5 mM Ca^{2+}, respectively. For measurement of DNA synthesis, cells were plated into 24-well plates (Costar) at a concentration of 10^4 cells per dish. After reaching confluence, cells were treated under the different conditions specified in the legends to the figures and labeled with [*methyl*-^{3}H]thymidine (78.6 Ci/mmol; Amersham) at a concentration of 4 μCi/ml for 4 h. After extensive washing, the cells were treated with 5% cold trichloroacetic acid, followed by 20 mM sodium hydroxide–1% sodium dodecyl sulfate (SDS), and counted in a scintillation counter.

RNA extraction and Northern (RNA) blot hybridization. Cells were plated in T-175 flasks (Falcon) at an initial concentration of 10^6 cells per flask and grown until subconfluent. Following various treatments as indicated in the legends to the figures, total cellular RNA was extracted by a modification of the guanidine hydrochloride method de-

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scribed elsewhere (1). RNAs (20 μg per lane) were denatured in 50% formamide and 2.2 M formaldehyde and fractionated by means of electrophoresis on 1% agarose gels containing 2.2 M formaldehyde and ethidium bromide (0.5 μg/ml). Gels were then subjected to partial alkaline hydrolysis in 50 mM sodium hydroxide for 30 min and subsequently to neutralization in 5× MOPS buffer (1× MOPS buffer is 20 mM 30'-[*N*-morpholino]propanesulfonic acid, pH 7.0, 5 mM sodium acetate, 1.0 mM EDTA) plus 1.1 M formaldehyde for 30 min and then in 1× MOPS buffer plus 1.1 M formaldehyde. RNAs were then transferred to nitrocellulose filters as described previously (41). Specific RNAs were then identified by hybridization with 2.0×10^6 cpm of nick-translated probes (5×10^8 cpm/μg, specific activity) per ml. Hybridizations were performed at 42°C for 24 h in 40% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), followed by washing at 48°C in 0.1× SSC–0.1% SDS, as described previously (22, 41). The probes used were a 1.1-kilobase (kb) *Pst*I-*Bgl*II fragment from a v-*fos* clone (19), a 5.5-kb *Bam*HI fragment from a mouse genomic clone of c-*myc* (26), a mixture of two 2.6- and 2.0-kb *Eco*RI fragments from a human genomic c-*myb* clone (16), a 0.8-kb *Ava*I-*Bam*HI fragment encoding the tyrosine kinase region of v-*fgr* (34), the 0.4-kb fragment from BS9 clone of v-Ha-*ras* and the 0.2-kb fragment from the HiHi3 clone of v-Ki-*ras* (14), a 1.5-kb *Bgl*II fragment from the cloned integrated form of v-*abl* (26), a mixture of two 0.5-kb fragments, *Bam*HI-*Bam*HI and *Bam*HI-*Eco*RI, from v-*erb* (44), a 0.9-kb *Xho*I-*Sac*II fragment from a cDNA clone of mouse p53 (48), and an exon II-containing *Sac*I-*Kpn*I fragment (540 base pairs) from a mouse genomic clone of β_2-microglobulin (35). Northern blots were checked for RNA content by being stripped, after hybridization, in 0.1% SDS at 90°C and rehybridized with a β_2-microglobulin probe. β_2-Microglobulin RNA levels did not change appreciably under any of the experimental conditions used.

RESULTS

Effect of EGF on BALB/MK cellular DNA synthesis and proto-oncogene expression. BALB/MK cells proliferate in the presence of EGF, whereas removal of this growth factor leads to cessation of growth (46). To investigate the kinetics of BALB/MK cell DNA synthesis in response to the removal of EGF as well as to its subsequent readdition, we measured [^{3}H]thymidine incorporation in appropriately treated BALB/MK cultures. DNA synthesis was reduced to undetectable levels within 48 h following removal of EGF from the culture medium (Fig. 1A). A synchronous peak of DNA synthesis associated with an increase of 100-fold above control levels of thymidine incorporation followed EGF addition. The peak occurred at around 20 h, with a detectable increase in DNA synthesis measurable as early as 12 h (Fig. 1A). Thymidine incorporation, as determined by autoradiography, indicated that greater than 95% of EGF-treated BALB/MK cells incorporated [^{3}H]thymidine into DNA during the first 24 h following the EGF stimulus (data not shown).

In an effort to analyze early events in the induction of proliferation in this synchronized epithelial cell population, we examined the expression of various proto-oncogenes as a function of time following EGF exposure. Certain of these genes have been shown to be activated at early times following stimulation of fibroblasts or lymphoid cells with platelet-derived growth factor (PDGF) (20, 27, 33) or phytohemagglutinin (25), respectively. The most dramatic effect on proto-oncogene expression was observed with c-*fos* (Fig. 1B). A marked increase in c-*fos* RNA levels was detectable within 15 min, with a peak of greater than a 100-fold increase at 1 h. The expression of c-*myc* also was found to increase as a result of EGF treatment. However, this increase occurred much later and less dramatically than observed with c-*fos* (Fig. 1B). Expression of a number of other proto-oncogenes

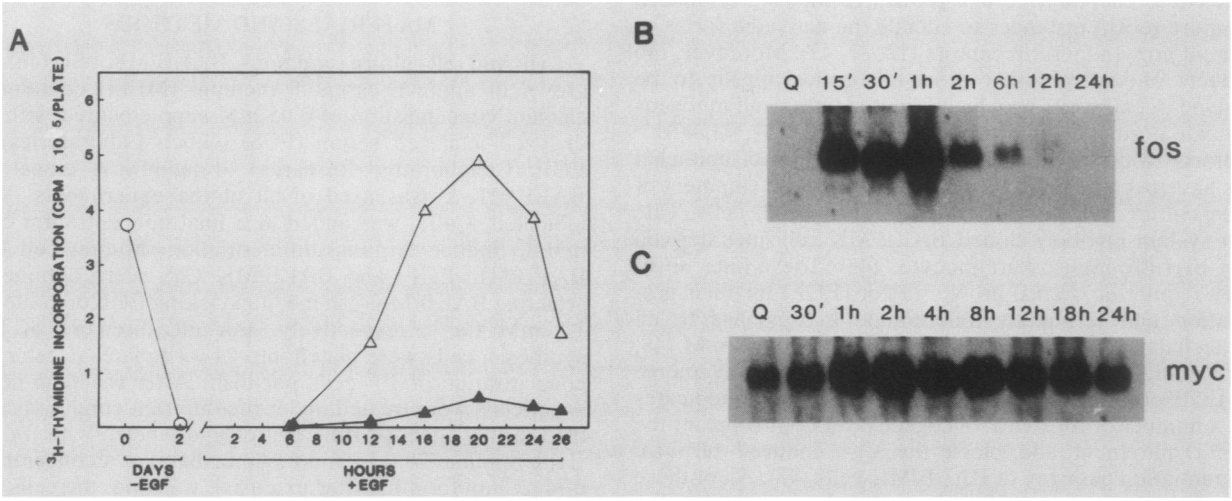

FIG. 1. Time course of proto-oncogene mRNA expression in synchronized BALB/MK cells following EGF stimulation. (A) BALB/MK cells were grown to confluence in MEM containing FCS and EGF (4 ng/ml), followed by removal of EGF from the medium (○). After 2 days, the cells were washed several times in serum-free MEM, and new medium containing either FCS (△) or FCS and EGF (4 ng/ml) (▲) was added. DNA synthesis was monitored by [^{3}H]thymidine incorporation at the time points indicated. (B and C) To measure c-*fos* and c-*myc* mRNA expression, RNAs were extracted from quiescent BALB/MK cells after 2 days of EGF starvation (Q) or at the indicated time points after EGF readdition. RNA blotting and hybridization with v-*fos* and mouse c-*myc* DNA probes were performed as indicated in Materials and Methods. The sizes of the c-*fos* and c-*myc* mRNAs are 2.2 and 2.3 kb, respectively. RNA content in each lane was standardized by stripping the blot in 0.1% SDS at 90°C and rehybridizing with a β_2-microglobulin probe (as described in Materials and Methods) in this and all subsequent figures. β_2-Microglobulin RNA levels did not change appreciably under any of the experimental conditions used (data not shown). Autoradiography was performed for 2 h for the *fos* blot (B) and 6 h for the *myc* blot (C).

was either not detectable, as was the case for c-*myb* and c-*fgr*, or did not show any detectable change, as with c-Ha-*ras*, c-Ki-*ras*, c-*abl*, c-*erb*B, and p53 (data not shown).

The rapid increase in c-*fos* RNA levels in EGF-stimulated BALB/MK cells resembled that reported for fibroblasts stimulated by PDGF (20, 27, 33). Moreover, as previously observed with PDGF-stimulated fibroblasts (33), simultaneous exposure of EGF-stimulated BALB/MK cells to cycloheximide resulted in enhanced induction of c-*fos* RNA levels. Expression of c-*fos* RNA under these conditions ranged from three- to fivefold above that observed following EGF treatment alone (data not shown).

High Ca^{2+} rapidly inhibits c-*fos* RNA expression. Exposure of BALB/MK-2 cells to 1.5 mM Ca^{2+} results in their terminal differentiation within 7 to 10 days. Cornified envelopes, a marker of terminally differentiated keratinocytes, can be detected as early as 3 days (46, 47). We searched for early markers of the Ca^{2+}-induced terminal differentiation by investigating both the time course of inhibition of DNA synthesis and early effects on proto-oncogene expression.

DNA synthesis in BALB/MK cells exposed to 1.5 mM Ca^{2+} decreased more than 90% within 2 days and was completely abolished by 4 days (Fig. 2A). The most rapid and striking effect of high Ca^{2+} was observed on c-*fos* RNA expression. We detected a greater than 80% decrease in c-*fos* RNA within 2 h of Ca^{2+} exposure (Fig. 2B). c-*myc* RNA levels were practically unchanged over the same time period (Fig. 2B). Other proto-oncogenes tested, including c-*myb*, c-*erb*B, and p53, also exhibited no detectable alteration in their expression (data not shown). The rapid reduction in c-*fos* RNA following high Ca^{2+} exposure was unlikely to simply reflect decreased cell proliferation, since it preceded any detectable change in the level of BALB/MK cell DNA synthesis.

High Ca^{2+} counteracts the influence of EGF at an early point in the proliferation pathway. The rapid and dramatic effects of both EGF and Ca^{2+} on c-*fos* RNA expression made it possible to inquire whether Ca^{2+} might intervene at a very early stage in the EGF-induced proliferative pathway. To address this question, we synchronized BALB/MK cells by EGF starvation and then exposed the cells to EGF with or without high Ca^{2+}. Both treatments were associated with a synchronous burst of cell DNA synthesis (Fig. 3A). However, concomitant exposure to high Ca^{2+} reduced the magnitude of DNA synthesis by ca. threefold (Fig. 3A). When we compared the levels of c-*fos* mRNA under each condition at 30 and 60 min following exposure to EGF, we observed that high Ca^{2+} exposure substantially reduced the magnitude of c-*fos* RNA stimulation observed with EGF (Fig. 3B). Moreover, this reduction appeared to correlate well with the diminished peak of BALB/MK cellular DNA synthesis observed at 20 h.

Treatment of quiescent BALB/MK cells with Ca^{2+} alone in the absence of EGF had little if any effect on c-*fos* mRNA (data not shown). The ability of Ca^{2+} to inhibit EGF-induced *fos* expression in the 30 min of simultaneous exposure implies a rapid counteraction of EGF very early in the EGF-induced cascade leading to DNA synthesis. We also examined whether Ca^{2+} must be added simultaneously with EGF to exert its inhibitory effects. For this purpose, quiescent BALB/MK cells were first incubated with high Ca^{2+} alone for up to 4 h and then washed free of Ca^{2+} and exposed to EGF for 30 min (Fig. 3C). Under these conditions, Ca^{2+} pretreatment had little if any effect on the EGF-induced increase in *fos*-specific mRNA levels. These results indicate that the Ca^{2+} inhibition of *fos* induction by EGF required the presence of both of these agents.

Spontaneous BALB/MK mutants with altered responses to either EGF or high Ca^{2+}. To investigate further the biological significance of c-*fos* RNA modulation during BALB/MK cell proliferation and differentiation, we attempted to select spontaneous mutants capable of growth either in the absence

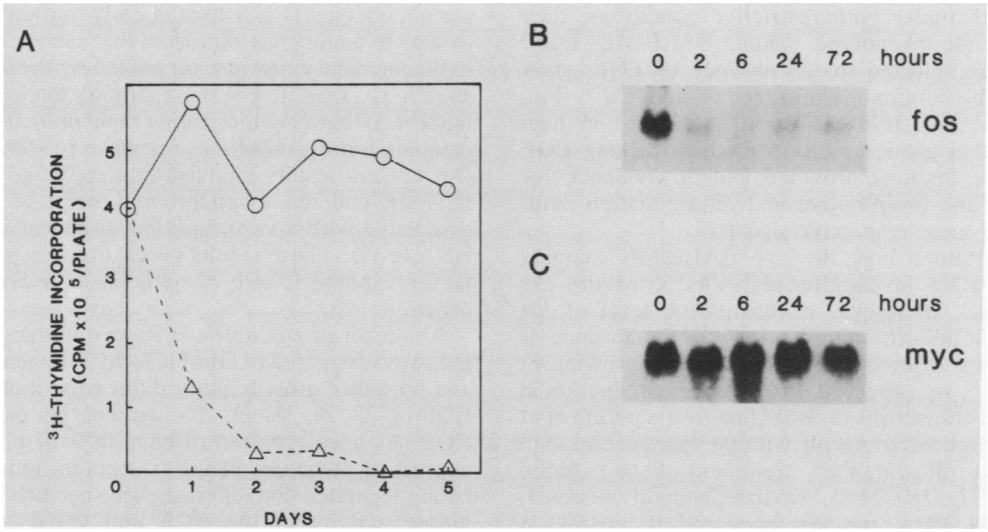

FIG. 2. Time course of proto-oncogene mRNA expression during Ca^{2+}-induced differentiation of BALB/MK cells. (A) BALB/MK cells, grown to confluence in MEM containing FCS and EGF (4 ng/ml), were washed several times in serum-free MEM (day 0), followed by the addition of medium containing FCS and EGF (4 ng/ml) (○) or FCS, EGF (4 ng/ml), and 1.5 mM $CaCl_2$ (△). DNA synthesis was monitored by [^{3}H]thymidine incorporation at the time points indicated. (B and C) To measure c-*fos* and c-*myc* mRNA expression in BALB/MK cells undergoing differentiation, RNAs were extracted from the cells at time zero or at the time points indicated after the addition of 1.5 mM $CaCl_2$. Lane O shows the steady-state *fos* mRNA level in the late exponential phase of BALB/MK cells continuously grown in the presence of EGF. The other lanes show the steady-state mRNA levels in parallel cultures grown in the presence of EGF and then exposed simultaneously to EGF and high Ca^{2+} for different lengths of time. RNA blotting and hybridization were performed as indicated in the legend to Fig. 1. Rehybridization to a β_2-microglobulin probe showed no appreciable changes in β_2-microglobulin-specific mRNA levels under any of the experimental conditions used (data not shown). Autoradiography was performed for 48 h for the *fos* blot (B) and 6 h for the *myc* blot (C).

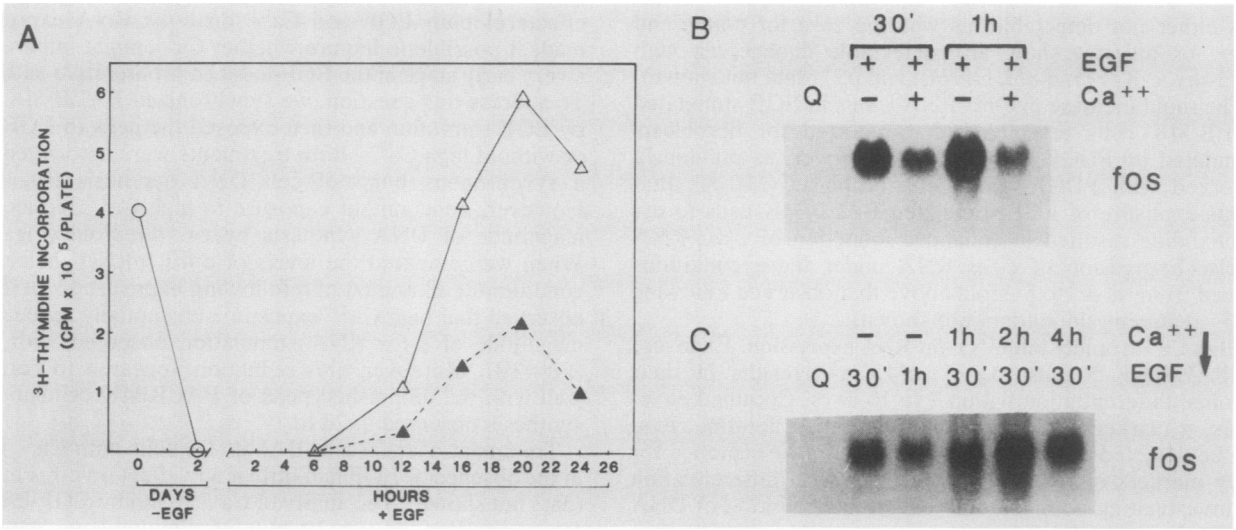

FIG. 3. Effect of high calcium on EGF-induced BALB/MK cell DNA synthesis and c-*fos* mRNA expression in BALB/MK cells. (A) BALB/MK cells, starved of EGF (○) as indicated in the legend to Fig. 1, were exposed to medium containing FCS and EGF (4 ng/ml) (△) or FCS, EGF (4 ng/ml), and 1.5 mM $CaCl_2$ (▲). DNA synthesis was monitored by [^{3}H]thymidine incorporation at the time points indicated. (B) The effect of high Ca^{2+} on c-*fos* mRNA expression was determined by extracting RNAs from quiescent BALB/MK cells starved of EGF for 2 days (Q) or at 30 min and 1 h after the addition of EGF in the absence (−) or presence (+) of 1.5 mM $CaCl_2$. RNA blotting and hybridization were performed as indicated in the legend to Fig. 1. Autoradiography was performed for 2 h. (C) BALB/MK cells were starved of EGF for 2 days (Q) and then treated with 1.5 mM $CaCl_2$ for 1, 2, or 4 h. Cells were then washed free of $CaCl_2$, and medium containing FCS and EGF (4 ng/ml) was added again for 30 min or 1 h. RNA blotting and hybridization were performed as indicated in the legend to Fig. 1. Autoradiography was performed for 2 h. The blots in both B and C were stripped and rehybridized with a β_2-microglobulin probe. By using this control probe, no appreciable difference in specific mRNA levels was detected under any of the experimental conditions used (data not shown).

of EGF or in the continuous presence of a high Ca^{2+} concentration. Such spontaneous mutants occurred only rarely. Of 3×10^7 cells plated, we were able to isolate one mutant which was independent of EGF for its growth (BALB/MK EGF^I) and one mutant capable of growth in 1.5 mM Ca^{2+} (BALB/MK Ca^r cells). When each mutant was tested for growth under both restrictive conditions, they displayed the same phenotype. Thus, BALB/MK EGF^I cells, which were selected in the absence of EGF, grew equally well in the continuous presence of high Ca^{2+} (Fig. 4A). Conversely, BALB/MK Ca^r cells, selected in high Ca^{2+}, were able to grow readily in medium lacking EGF (Fig. 4A). These findings provided genetic evidence for linkage between the proliferative and differentiation pathways induced by these respective signals.

We next ascertained how the two BALB/MK mutants modulated c-*fos* RNA levels after high Ca^{2+} exposure. As shown in Fig. 4B, there was a constitutive level of *fos* mRNA detectable in both mutants grown in the absence or presence of EGF. After exposure of cultures growing in EGF to high Ca^{2+}, we observed no appreciable reduction in the levels of c-*fos* transcripts in either line over a period of at least 72 h. This contrasted sharply with the rapid decrease in *fos* mRNA levels following Ca^{2+} treatment of the parental BALB/MK line (Fig. 2B). Thus, two independently selected, spontaneous BALB/MK mutants appeared to possess a genetically linked alteration in both their responsiveness to Ca^{2+}-induced terminal differentiation and their EGF requirement for proliferation. Moreover, this alteration in each case could be correlated with an altered modulation of c-*fos* RNA expression.

DISCUSSION

Accumulating evidence has linked a small subset of genes involved in normal cellular proliferation to the neoplastic process. Genes coding for a normal growth factor (10, 45) and for two different growth factor receptors (12, 37–43) have acquired transforming properties when transduced by acute transforming viruses. Indirect evidence linking other oncogenes to the same pathways has come from a number of approaches. Evidence that the expression of genes related to certain oncogenes is activated early in the cellular response to growth factors has suggested that such genes are involved in biochemical pathways stimulated by these mitogens (5–7, 20, 27, 31, 33, 42). The most striking findings in this regard have been those demonstrating rapid induction of c-*fos* RNA of quiescent fibroblasts in response to PDGF (20, 33, 37). Our present results establish that epithelial cells stimulated by EGF undergo a synchronous burst of DNA synthesis associated with a very rapid but transient increase in c-*fos* RNA levels. These results generalize this pattern of growth factor response to cells of epithelial as well as mesenchymal origin.

A number of studies have suggested that the intracellular pathways triggered in fibroblasts by mitogens such as PDGF and fibroblast growth factor differ from those stimulated by EGF (8, 28, 29, 38, 39). For example, in mouse fibroblasts, EGF has been reported to have little or no effect on c-*fos* expression, whereas PDGF is a potent inducer (27). It has been speculated that differences in specificities of the protein kinase activity of the EGF and PDGF receptors might account for the different actions of these growth factors on fibroblasts (27). Thus our results and previously published findings indicate that tissue- or cell-specific differences of a point in the signalling pathway distal to the receptor itself may also account for the very different effects of EGF on c-*fos* expression in fibroblasts and epithelial cells.

In certain cell types, optimal proliferation appears to be incompatible with the fully differentiated phenotype (3, 18, 19, 21, 46, 47). The high Ca^{2+} signal for terminal differenti-

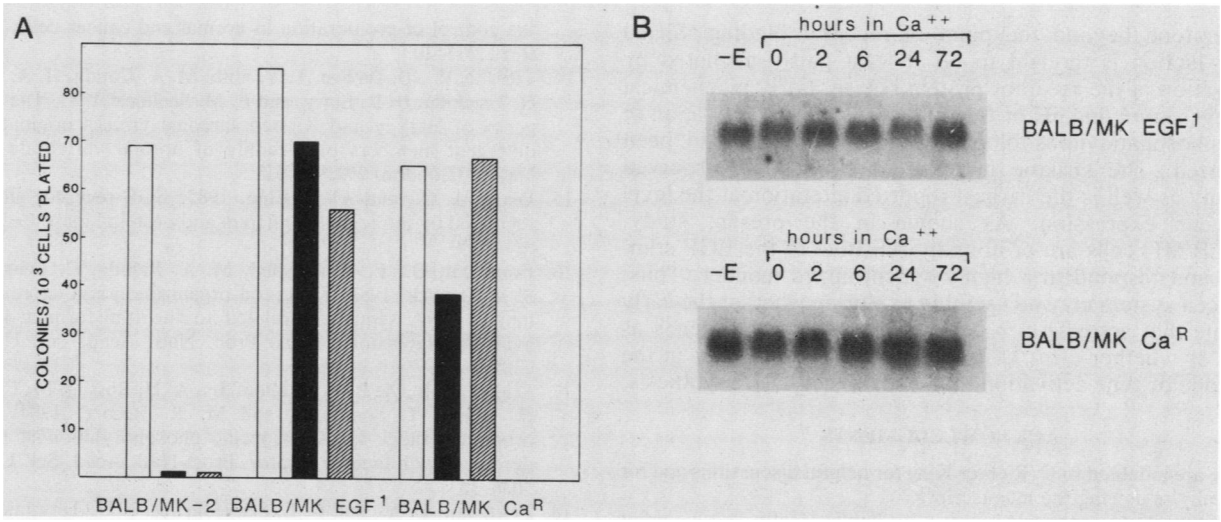

FIG. 4. Growth properties and c-*fos* mRNA expression in spontaneous BALB/MK mutants exposed to high Ca^{2+}. (A) Parental BALB/MK and the spontaneous mutants obtained as described in Materials and Methods were compared for their ability to grow in the presence of FCS and EGF (4 ng/ml) (open bars), FCS alone (solid bars), or FCS, EGF (4 ng/ml), and 1.5 mM $CaCl_2$ (striped bars). Cells were plated at serial 10-fold dilutions in medium containing FCS and EGF (4 ng/ml). Following overnight incubation, the cells were refed as indicated. At 2 weeks, petri dishes were ethanol fixed and Giemsa stained. Colonies containing more than 100 cells were scored as positive. The results reflect the mean of two independent experiments. (B) To measure c-*fos* mRNA expression in spontaneous mutant BALB/MK lines exposed to high Ca^{2+}, RNAs were extracted from cells grown in the absence of EGF in medium plus FCS (−E lanes) or in medium containing EGF (4 ng/ml) and FCS and supplemented with 1.5 mM $CaCl_2$ for various lengths of time. Lanes O represent RNAs from cells grown in the presence of EGF and extracted prior to exposure to high Ca^{2+}. The other lanes represent RNAs extracted from cells grown in the presence of EGF and then exposed to high Ca^{2+} for the indicated length of time. RNA blotting and hybridization were performed as indicated in the legend to Fig. 1. Rehybridization to a β_2-microglobulin probe showed no appreciable differences in β_2-microglobulin-specific mRNA levels under any of the experimental conditions (data not shown). Autoradiography was performed for 12 h.

ation of BALB/MK cells was associated with a profound decrease in cellular DNA synthesis within 48 h, well before the cells exhibited markers of terminally differentiated cells (46, 47). Moreover, these effects of high Ca^{2+} were demonstrated in the presence of continued cell exposure to EGF. The ability of high Ca^{2+} to overcome the EGF proliferative signal was found to correlate well with its ability to inhibit the increase in c-*fos* RNA in response to EGF. At the present we cannot discriminate whether EGF or Ca^{2+} acts on *fos* mRNA transcription or by a mechanism involving decreased *fos* mRNA stability, or whether both mechanisms may be involved. Nevertheless, our results are consistent with the possibility that both the EGF proliferative and high Ca^{2+} differentiation signals exert at least part of their actions through their signaling of a common pathway that modulates c-*fos* expression. The very rapid effects observed further establish that this potent differentiation signal must act at a very early stage to inhibit the proliferation process.

Despite up to 4 h of pretreatment, removal of Ca^{2+} prior to EGF exposure led to no detectable inhibition of *fos* induction. Thus, Ca^{2+} treatment had to be simultaneous with EGF in order to be effective. These findings imply either that the Ca^{2+} effect must be very labile or that very early events in EGF stimulation are needed to make available the necessary substrates for the Ca^{2+}-mediated inhibition of *fos* RNA expression.

It is known that Ca^{2+} can activate protein kinase C (15, 24, 40), which in turn can reduce the ability of EGF to bind to the EGF receptor (15), probably through phosphorylation of the receptor (15, 17, 23). The kinetics of inhibition of DNA synthesis in response to high Ca^{2+} corresponded to the time course of cessation of DNA synthesis following removal of EGF from proliferating BALB/MK cells. Thus, it is possible that high Ca^{2+} may act to inhibit c-*fos* RNA expression at the level of the EGF receptor itself. In any case, the ability of high Ca^{2+} to induce terminal differentiation is unlikely to be accounted for solely by inhibition of cell proliferation. BALB/MK cultures from which EGF was removed for several days could still be rescued by EGF readdition, whereas exposure to high Ca^{2+} led to an irreversible block to proliferation during the same time period (P. P. Di Fiore, unpublished observations). The existence of multiple intracellular pathways mediating keratinocyte differentiation in vitro is also suggested by a recent report from Dotto et al. (11), who found a different response of c-*fos* to the differentiation inducers $CaCl_2$ and 12-*O*-tetradecanoylphorbol-13-acetate.

A number of oncogenes have been shown both to induce EGF-independent proliferation of BALB/MK cells and to block their terminal differentiation in response to high Ca^{2+} (46, 47). Thus, mechanisms that "short-circuit" the normal growth factor activation of cell proliferation appear to be incompatible with BALB/MK cell terminal differentiation. In the present studies, we were able to obtain rare, spontaneously occurring BALB/MK mutants which were selected for either Ca^{2+} resistance or EGF independence. We found that both mutants displayed the dual phenotype: each was both Ca^{2+} resistant and EGF independent. These findings provide further evidence that the proliferative and differentiation pathways are somehow linked in keratinocytes. Our findings further imply that alteration of EGF and Ca^{2+} regulation of c-*fos* RNA levels in the mutants provides a good marker of their altered phenotype. Whether these mutants contain alterations that affect other known proto-oncogenes or some yet to be identified gene remains to be determined.

Very early events in the activation of cell DNA synthesis in response to growth factors remain for the most part poorly

understood beyond the point of receptor binding. Signal transduction is thought to be at least partly mediated by activation of the receptor protein kinase. Early biochemical changes involving pH or Ca^{2+} flux (13, 32) and activation of the phosphatidylinositol pathway (2, 30) have also been reported. The striking increase in c-*fos* RNA observed rapidly as well is the earliest reported alteration at the level of gene expression. As shown in the present study, BALB/MK cells are profoundly sensitive to the EGF stimulus and respond in a highly synchronized manner. Thus, this cell system may be useful in exploring whether the early events that activate c-*fos* expression affect other genes as well or whether c-*fos* is itself the major early target in the cascade of gene activation that leads to cell DNA synthesis.

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