c-myc and c-fos expression in differentiating mouse primary keratinocytes

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Expression of the *myc* and *fos* genes has been monitored in mouse primary keratinocytes after induction of terminal differentiation by calcium or tetradecanoylphorbol acetate (TPA). *myc* RNA levels in growing cells are very high and remain elevated even at late times after calcium-induced differentiation. Thus, keratinocytes provide the first example of normal primary cells with persistent *c-myc* expression irrespective of their proliferative or differentiated state. *fos* expression is also relatively unaffected by addition of calcium. In contrast to calcium, TPA-induced differentiation is accompanied by dramatic changes in proto-oncogene expression: marked *c-fos* induction and considerable although transient decrease in *c-myc* expression. These effects might be important for the keratinocyte response to TPA: TPA treatment of a keratinocyte cell line (RBK) resistant to this substance has no effect on *c-myc* expression and leads only to minimal *c-fos* induction. In these cells full *fos* induction can still be triggered by addition of fresh medium. Thus, the *fos* gene in normal keratinocytes is inducible through at least two independent mechanisms, only one of which has been lost during derivation of the TPA-resistant cell line.

Key words: epithelial cell differentiation/*fos*/*myc*/primary keratinocytes/tetradecanoylphorbol acetate (TPA)

Introduction

Mouse primary keratinocytes provide a good model to investigate epithelial growth and differentiation (Hennings *et al.*, 1980). These cells can be grown in low calcium medium as basal, relatively undifferentiated cells and can be rapidly and efficiently induced to terminally differentiate by increase in the level of calcium in the culture medium (Hennings *et al.*, 1980). As shown by Yuspa and co-workers, a close parallel can be established between disturbances in this differentiation program and the onset of skin carcinogenesis (Yuspa and Morgan, 1981; Kilkenny *et al.*, 1985). Keratinocyte differentiation by calcium in culture is only a reflection of the more complex and ordered process occurring *in vivo* (see, for instance, Fusenig *et al.*, 1983). For this reason the term 'keratinocyte differentiation' will be used in this paper only in a very restricted sense, referring to the set of inducible phenomena in keratinocytes in the presently described culture system.

Expression of the cellular *myc* and *fos* proto-oncogenes has been found to be tightly regulated and coupled to the control of growth and differentiation of a variety of cell types. Mitogenic treatment of fibroblasts and lymphocytes results in a rapid and transient increase in *c-myc* and *c-fos* mRNA (Kelly *et al.*, 1983; Cochran *et al.*, 1984; Greenberg and Ziff, 1984; Kruijer *et al.*,

1984; Müller *et al.*, 1984a). In other types of cultured cells in which terminal differentiation can be induced, *c-myc* RNA levels decrease substantially prior to the arrest of DNA synthesis (Westin *et al.*, 1982; Campisi *et al.*, 1984; Endo and Nadal-Ginard, 1986), while *fos* expression is induced and then rapidly reverts to its basal level (Müller *et al.*, 1984b, 1985; Curran and Morgan, 1985; Kruijer *et al.*, 1985; Mitchell *et al.*, 1985).

We have studied *myc* and *fos* expression in keratinocytes as a first attempt to understand the mechanisms underlying proliferation and differentiation of these cells. We have cultivated primary keratinocytes from BALB/c mice and derived keratinocyte cell variants that are resistant to induction of differentiation by calcium or tetradecanoylphorbol acetate (TPA). In this way, we have been able to study *c-myc* and *c-fos* expression in primary keratinocytes either under growing conditions or upon induction of differentiation and we could compare the responses of these genes in the primary cells with those of the *myc* and *fos* genes in a keratinocyte cell line resistant to calcium- and TPA-induced differentiation.

Results

Derivation of primary keratinocytes and differentiation-resistant variants

Primary mouse keratinocytes proliferate actively when kept in medium with low calcium concentration (0.05 mM; low calcium medium) as described by Hennings *et al.* (1980). Terminal differentiation of all of these cells in culture can then be induced by increasing the calcium concentration in the medium to 2 mM (high calcium medium) (Hennings *et al.*, 1980). Upon prolonged cultivation in high calcium medium, however, cell variants can be derived that are resistant to calcium-induced differentiation and grow actively even in high calcium medium (Kulesz-Martin *et al.*, 1980; Kilkenny *et al.*, 1985). The RBK cell line is a keratinocyte cell line that we derived from a culture of primary keratinocytes of BALB/c mice by selection in high calcium medium. The keratinocyte nature of the RBK cells was confirmed

Table 1. Differentiation parameters of primary and RBK cells

	DNA synthesis (% of the control)		Cornified envelopes (% of the total cell number)	
	BALB/c	RBK	BALB/c	RBK
High calcium 24 h	2%	>100%	3%	<1%
High calcium 5 days	0.2%	100%	20%	3%
TPA 24 h	20%	100%	15%	<1%

DNA synthesis was measured by [³H]thymidine incorporation. Cells were pulse labelled with [³H]thymidine (1 µCi/ml, 78 Ci/mmol, NEN) for 1 h, DNA was prepared by treatment with NaOH (0.2 M) for 10 min at 4°C and TCA-precipitable counts were determined and normalized against DNA content of each sample [measured in a H33258 fluorescent assay (Labarca and Paigen, 1980)]. Cornified envelopes were measured by trypsinizing cells, washing in PBS and incubating at 68°C in 1% SDS, 0.1% β-mercaptoethanol for 10 min. The number of cells prior to and after treatment was determined.

Fig. 1. *myc* and *fos* RNA expression in either primary keratinocytes or calcium-resistant cells (RBK) upon increase of calcium concentration in the medium. Total cellular RNA was obtained from RBK cells or primary cells kept in culture for 2 weeks in low calcium (0.05 mM) medium and switched to high calcium (2 mM) at various times; control in low calcium: 1; high calcium for 30 min: 2; for 2 h: 3; for 24 h: 4; for 5 days: 5; RNA from resting BALB/c 3T3 cells serum-stimulated for 1 h: 6; RNA from quiescent BALB/c 3T3 cells: 7; tRNA control: C; size marker: M. RNA (10 µg) was analysed by ribonuclease protection assay (Melton *et al.*, 1984) using an SP6 probe containing sequences derived either (a) from the mouse *c-myc* first intron and second exon or (b) both from the mouse *c-myc* first intron and second exon and from the *c-fos* gene. The RNAs analysed in a and b derive from two independent experiments.

by keratin analysis that revealed a pattern identical to that of primary cells (data not shown).

Primary keratinocyte cultures from BALB/c mice and RBK cells were grown to confluence in low calcium medium. The calcium concentration of the medium was then increased to 2 mM. As previously described by others (Hennings *et al.*, 1980), the resulting induction of differentiation in the primary cells led to rapid morphological changes and an almost complete arrest of DNA synthesis by 24 h (Table I). Formation of SDS-resistant cornified envelopes (Sun and Green, 1976) was another result of differentiation (Table I). As expected, the calcium-resistant RBK cells continued to grow, showing no arrest of DNA synthesis and no or little formation of cornified envelopes (Table I).

myc and *fos* expression in response to calcium

Total cellular RNA was prepared from primary keratinocytes and RBK cells at various times after calcium addition and analysed by SP6-ribonuclease protection assay (Melton *et al.*, 1984), using probes specific for either the *myc* or the *fos* genes. The levels of *myc* mRNA found both in proliferating keratinocytes (in low

calcium medium) and in RBK cells were significantly higher than in fibroblasts in which substantial *myc* expression had been induced by a 1 h exposure of previously quiescent cells to serum (Figure 1a, lanes 1 and 6, 7).

myc expression in the primary keratinocytes remained substantial even after induction of differentiation. In some experiments, it was reduced imperceptibly (Figure 1a), while in others (Figure 1b) levels of *myc* decreased ~3-fold by 24 h after calcium addition. This lower level of *myc*, seen in cells where DNA synthesis is reduced to <5% of the control (Table I), is still several fold higher than that seen in fibroblasts induced to grow by serum addition (cf. Figure 1a and b). Indeed, *myc* RNA levels remained very high even 5 days after calcium exposure (Figure 1a, lane 5), under conditions where DNA synthesis was completely arrested (Table I). Similarly, no difference in *myc* RNA levels could be detected between freshly isolated keratinocytes, prior to plating and containing a large fraction of differentiated cells, and actively replicating keratinocytes kept in culture in low calcium medium for 2 weeks (data not shown). The pattern of *myc* expression in response to calcium was similar in primary keratinocytes and RBK cells (Figure 1a and b).

These findings differ from those observed with most other cell types studied to date, which suggest some linkage between expression of *myc* and cellular growth and differentiation (Kelly *et al.*, 1983; Campisi *et al.*, 1984; Greenberg and Ziff, 1984). One exception stems from tumor cells of the PC12 pheochromocytoma line which exhibit a response related to that seen here: upon induction of differentiation, a brief peak of *myc* expression is followed by a lower level of sustained expression, long after cell proliferation has stopped (Greenberg *et al.*, 1985). However, it is unclear whether this behavior reflects that of the normal counterparts of the PC12 cells or is a consequence of the malignant state of these cells.

A number of cell types exhibit a sharp increase in *fos* expression in response to agents that induce their differentiation (Müller *et al.*, 1984, 1985; Curran and Morgan, 1985; Greenberg and Ziff, 1984; Kruijer *et al.*, 1985; Mitchell *et al.*, 1985). In contrast, keratinocyte differentiation triggered by calcium is not accompanied by induction of *fos* expression (Figure 1b). Instead, the low levels of *fos* RNA present inside the cell undergo a slight decrease by 2 h after exposure to calcium (Figure 1b). This pattern of *fos* expression was observed with both primary and RBK cells (Figure 1b). This suggests that *fos* activation might not be an essential element of the mechanism involved in triggering keratinocyte differentiation by calcium.

myc and *fos* expression in response to TPA

The tumor promoter TPA induces terminal differentiation in a large proportion of primary keratinocytes in culture in a manner similar to calcium (Yuspa *et al.*, 1982, 1983; Table I). Cells such as those of the RBK line, which have been selected for resistance to calcium, are also found to be resistant to TPA-induced differentiation (Yuspa *et al.*, 1983; Table I). This might indicate that the two inducers act through a common pathway.

Unexpectedly, the effects of TPA on *myc* and *fos* expression in the primary keratinocytes differed dramatically from those previously observed with calcium. Unlike the persistent expression of *myc* RNA seen upon addition of calcium, TPA addition led to a marked decrease (>10-fold) in *myc* RNA levels within 2 h of exposure (Figure 2). This decrease in *myc* RNA was specific for this RNA and was not detected for other mRNAs such as tubulin mRNA (data not shown). By 24 h after TPA addition, when DNA synthesis was diminished to <20% of the control (Table I), *myc* RNA rose again to elevated levels (Figure 2). The

Fig. 2. *fos* and *myc* RNA expression in either primary keratinocytes or RBK cells upon treatment with TPA (100 ng/ml). RNAs were prepared and analysed simultaneously for *fos* and *myc* as described in Figure 1. DMSO control (0.1%) for 30 min: 1; DMSO control for 2 h: 2; TPA for 30 min: 3; TPA for 2 h: 4; TPA for 24 h: 5.

late rise in *myc* expression further demonstrates that in the keratinocyte, regulation of *myc* expression and DNA synthesis are uncoupled.

TPA elicited a *fos* response quite different from that seen after application of calcium. After TPA treatment, *fos* RNA levels underwent a considerable (>30-fold) and transient increase. Maximal expression was reached by 30 min after TPA addition and remained very high for at least 2 h (Figure 2). However, by 24 h *fos* expression reverted to basal levels (Figure 2).

Taken together, our results with *myc* and *fos* expression suggest that calcium and TPA induce keratinocyte differentiation by two related but distinct pathways. This conclusion is supported by the different morphological changes induced by calcium and TPA (not shown) and by the uniform response of all primary keratinocytes in a culture to calcium-induced differentiation (Hennings *et al.*, 1980), this contrasting with the incomplete response of keratinocyte populations to treatment with TPA (Yuspa *et al.*, 1982; Parkinson *et al.*, 1984).

The strong *fos* response observed with TPA and the absence of a similar response with calcium is reminiscent of that found with HL60 promyelocytic leukemia cells. There, TPA treatment triggers monocytic differentiation and causes a marked and sustained *fos* induction (Müller *et al.*, 1984b, 1985; Mitchell *et al.*, 1985), while retinoic acid and dimethyl sulphoxide (DMSO) lead to granulocytic differentiation with no effect on *fos* expression (Mitchell *et al.*, 1985). Thus, the nature of the *fos* response

Fig. 3. Differential *fos* induction in RBK cells by fresh medium or TPA. Total cellular RNA from RBK cells was analysed simultaneously for *fos* and *myc* as described in Figure 1. Control: 1; 30 min after addition of fresh medium (0.05 mM calcium): 2; 30 min after addition of TPA (100 ng/ml): 3.

depends upon the inducing agent used and the type of differentiation pathway that it triggers. These, and more recent results in HL60 cells (Mitchell *et al.*, 1986), suggest that modulation of *fos* expression in these myeloid cells, as well as in the presently described keratinocytes, is not tightly coupled to the differentiation programs of these cells.

The availability of RBK cells, which are resistant to calcium and TPA-induced differentiation, prompted us to examine how *myc* and *fos* expression in these cells is affected by TPA. In contrast to the TPA-induced reduction of *myc* in the primary cultures, *myc* gene expression in RBK cells was unaffected by TPA treatment (Figure 2). *fos* expression was similarly unresponsive: it was only slightly stimulated by TPA, in contrast to the enormous increase seen in the primary cells (Figure 2). This slight increase in *fos* expression persisted in the RBK cells even 24 h after TPA addition. We conclude that, as a consequence of a selection against calcium-induced differentiation, the RBK cells have also acquired resistance to TPA-induced differentiation and have lost the normally observed regulation of the *fos* and *myc* genes by TPA. This situation is reminiscent of the block in *fos* expression recently described for some TPA-resistant variants of the HL60 cell line (Mitchell *et al.*, 1986).

The marked decrease of *fos* inducibility by TPA in RBK cells led us to test whether the *fos* gene of these cells can still be regulated by serum, which serves as a strong *fos* inducer in primary keratinocytes (data not shown) as well as in fibroblasts (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Müller *et al.*, 1984a). Indeed, as is seen in Figure 3, the *fos* gene of RBK cells remains highly responsive to serum, increasing 20-fold by 30 min after serum addition. This allows the additional conclusion that the *fos* gene in the keratinocytes can be induced through at least two distinct pathways, only one of which has been lost during the selection of the RBK cells. The second pathway would appear to be independent of the action of kinase C, the enzyme for which TPA serves as an agonist (Nishizuka, 1984).

Discussion

Mouse primary keratinocytes provide an ideal model-system to study epithelial growth and differentiation (Hennings *et al.*, 1980). An understanding of these phenomena at the molecular level is a necessary prerequisite for a better understanding of carcinogenesis, given the epithelial origin of the great majority of tumors. In a large variety of cells, *myc* and *fos* expression are found to be tightly linked to the control of growth and differentiation. Our analysis of the expression of these genes in the primary keratinocytes and in the RBK keratinocyte cell line has led to a few unexpected findings.

c-myc RNA expression in the keratinocytes is very high and remains so irrespective of the proliferative or differentiated state of the cell. The use of primary cells and not of an established cell line allows the conclusion that this persistent mode of *myc* expression is a true characteristic of the keratinocyte cell type and it is not an artifact due to establishment of cell lines in culture. In fact, establishment in culture is very likely to affect the molecular mechanisms regulating cell growth [such as, for instance, constitutive *myc* expression (Campisi *et al.*, 1984)]. This is especially true for mouse cells in general and keratinocytes in particular. Such cells are known to evolve rather rapidly in culture and undergo spontaneous transformation with relative ease (Fuse-nig *et al.*, 1983).

The different pattern of *c-myc* RNA expression in keratinocytes versus fibroblasts or lymphocytes could not be explained by structural differences in their *c-myc* genes (data not shown). Similarly, RNA blot analysis revealed a transcript in keratinocytes of size indistinguishable from that found in fibroblasts (data not shown). Our observations concerning *myc* expression in the keratinocytes are consistent with a report recently published by Yuspa and co-workers (Toftgard *et al.*, 1985).

fos and *myc* expression in the primary keratinocytes are not significantly modulated in response to calcium while they are dramatically affected by TPA. This shows that calcium and TPA, although inducing a similar differentiation program, elicit very different molecular effects on the primary keratinocytes. Whether the modulation in proto-oncogene expression triggered by TPA is important for induction of differentiation by this substance is not clear. However, the two processes appear to be co-regulated in that we find a substantial reduction in the *myc* and *fos* responsiveness to TPA in the keratinocytes (RBK cells) that have also lost their responsiveness to induced differentiation by this agent.

The resistance of the RBK cells to TPA is probably due to an early block in the cascade of events triggered by this substance. This is suggested by the fact that the markedly reduced inducibility of *fos* in the RBK cells is specific for TPA and by the fact that *fos* induction by TPA or other mitogens is a very early event for which no protein synthesis is required (Kruijer *et al.*, 1984; Müller *et al.*, 1984; Greenberg *et al.*, 1986). An elucidation of the molecular mechanism responsible for resistance to TPA could lead to an understanding of the concomitant acquisition of resistance to calcium, as it is observed in the RBK cells and in many other spontaneous or carcinogen-induced keratinocyte cell lines (Yuspa *et al.*, 1983; Hennings *et al.*, 1984; our unpublished observations).

Materials and methods

Cells

Primary keratinocytes were isolated from newborn BALB/c mice as described by Yuspa and Harris (1974) and cultivated in MEM medium with 8% Chelex-treated fetal calf serum (Hyclone), EGF (10 ng/ml; Collaborative Research) and

0.05 mM CaCl₂ as described by Hennings *et al.* (1980). TPA was purchased from Sigma and used in DMSO solution. Calcium and TPA were added directly to the cells without concomitant change of medium. This allowed us to show that *fos* expression is induced by addition of fresh medium, but not by calcium or, in the RBK cells, by TPA.

Ribonuclease protection assays

Total cellular RNA was isolated by the guanidinium isothiocyanate method (Chirgwin *et al.*, 1979). RNA levels were determined by an SP6 ribonuclease protection assay as described by Melton *et al.* (1985). Total cellular RNA (10 µg) was hybridized to 5 × 10⁵ d.p.m. probe RNA, labelled to 10⁹ d.p.m./µg, at 42°C overnight in the described buffer (Melton *et al.*, 1985). Ribonuclease digestions were carried out for 30 min at 37°C. Analysis was on 8% polyacrylamide, 50% (w/v) urea sequencing gels. The *c-fos*-specific SP6 probe contained sequences from -56 to +109 relative to the cap site (nucleotides 79–243 in Van Beveren *et al.*, 1983). The *c-myc*-specific probe spanned the junction of the first intron and second exon.

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