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Differential Induction of Transcription of c-*myc* and c-*fos* Proto-Oncogenes by 12-O-tetradecanoylphorbol-13-acetate in Mortal and Immortal Human Urothelial Cells

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The effect of the skin tumor-promoter TPA (12-O-tetradecanoylphorbol-13-acetate) on expression of cellular proto-oncogenes has been examined in cell lines derived from human urothelium. A single treatment with TPA (1 μg/ml) increased the transcription of c-*fos* and c-*myc* proto-oncogenes at least 20-fold in the mortal cell line HU 1752. The induction was transient and was accompanied by a rapid but transient change in cell morphology. When immortalized cell lines were treated with TPA a similar rapid and transient morphological response was observed, but the TPA treatment only increased the level of c-*fos* mRNA, suggesting that the normal regulation of c-*myc* transcription is altered in immortalized cells irrespective of their tumorigenic properties. The levels of c-Ha-*ras* and c-Ki-*ras* mRNAs were unaffected by TPA treatment in all cell lines.

Key words: TPA, oncogene expression, human epithelial cells, phorbol ester, immortalization, c-*myc*, c-*fos*

It is generally accepted that the development of cancer in man and experimental animals is a multistage process [1,2]. Evidence supporting this view has been obtained from the experimental induction of skin cancer in mice and bladder cancer in rats [3–5]. In these models the carcinogenic process has been separated into at least three different stages: initiation, promotion, and malignant conversion [6–8]. In our laboratory, a series of human urothelial cell lines has been developed that have been classified into three catagories: 1) mortal and nontumorigenic, 2) immortal but nontumorigenic, and 3) immortal, tumorigenic cell lines [19,20]. It has been suggested that these cell lines provide an in vitro model representing successive stages in multistage carcinogenesis [19].

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One of the most potent tumor-promoter substances in experimental animal models is the phorbolester 12-O-tetradecanoylphorbol-13-acetate (TPA) [9]. TPA as well as the naturally occurring mitogens platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) have been found to elicit transient transcription of two cellular oncogenes, c-*myc* and c-*fos*, in serum-starved rodent cells [10–12]. In contrast to these observations, we have previously reported that TPA increased c-*fos* expression but did not increase expression of c-*myc* in the immortalized, nontumorigenic human bladder epithelial cell line HCV 29 [13]. Since altered regulation of c-*myc* has been implicated in the genesis of tumors [17,18] we questioned whether the apparent resistance of c-*myc* to induction by TPA was correlated to the growth and tumorigenic properties of the cells. Accordingly, we have examined the effect of TPA on oncogene expression in human cell lines representing the mortal, the immortal, and the tumorigenic classes.

MATERIALS AND METHODS

Cell Lines and Growth Conditions

The cell lines used in this study are described briefly in Table I. The cells were serially propagated as previously described [19]. The mortal line HU 1752 was cultured in FIB41B medium [21] supplemented with seven nonessential amino acids (7NEA) and 20% fetal bovine serum (FBS). The immortalized cell lines were cultured in FIB41B medium supplemented with 7NEA and 5% FBS.

Oncogene Probes

By nick translation [22] ^{32}P-labelled DNA probes with a specific activity of approximately 10^8 cpm/μg were prepared. The probes were the 1.3-kb *Cla*I-*Eco*RI fragment of the human c-*myc*, the 2.4-kb *Eco*RI fragment of the human c-Ki-*ras*-2, the 2.8-kb *Sac*I fragment of the human c-Ha-*ras*-1, and the 1,050-bp *Pst*I fragment of the v-*fos* gene of FBJ murine osteosarcoma virus [23].

TPA Exposure

Cultures, approximately 80% confluent, were treated with a single dose of TPA dissolved in dimethyl sulfoxide (DMSO). The final concentrations of TPA and DMSO were 1 μg/ml and 0.1%, respectively. The cells were incubated with TPA at 37°C for various periods of time as indicated in Figures 2, 3, and 5.

RNA Isolation and Filter Hybridization

Total cellular RNA was prepared by a variation of the guanidine thiocyanate method by using a ten-times-higher amount of beta-mercaptoethanol than originally

TABLE I. Properties of Cell Lines

Cell line	Life span in vitro[a]	Tumorigenicity in nude mice[b]
HU 1752	Mortal	Nontumorigenic
HCV 29	Immortal	Nontumorigenic
HU 609	Immortal	Nontumorigenic
HCV 29T	Immortal	Tumorigenic
HU 609T	Immortal	Tumorigenic

[a]We define immortal as survival for more than 75 passages in vitro (equivalent to more than 200 population doublings).

[b]The tumorigenic properties were tested by subcutaneous inoculation of 10^7-10^8 trypan-blue-negative cells into 6–8 wk-old inbred BALB/c athymic nude mice as previously reported [19].

described [24]. The integrity of each RNA sample was confirmed by gel electrophoresis of glyoxylated RNA [25]. Only RNA samples showing intact 28S and 18S ribosomal bands with a 28S:18S ratio bigger than 2:1 were analysed further. The relative levels of oncogene-related RNA were determined by a modification of the dot blot technique [26,27] with a Minifold II apparatus and BA 85-3B nitrocellulose filter (Schleicher and Schuell, Dassel, West Germany). Hybridization of serial dilutions (10, 5, 2.5 μg) of RNA was performed at high stringency (50% formamide at 45°C) as described elsewhere [28,29]. The hybridization was followed by stringent washing 3× for 20 min in 2× SSC (1 × SSC-buffer is 0.15 M NaCl + 0.015 Na citrate), 0.1% sodium dodecylsulfate (SDS) at 50°C and 3× for 20 min in 0.1 × SSC, 0.1% SDS at 50°C. Autoradiograms were prepared by using Agfa Structurix D7 film (Agfa, Leverkusen, West Germany). This film shows a linear response to ^{32}P without preflashing.

RESULTS

When human urothelial cells cultured in vitro were treated with a single dose of TPA (1 μg/ml), a rapid change in morphology was observed (Fig. 1). This change was seen in all cell lines regardless of their life span in vitro. The effect of TPA on the cell morphology was transient. The change in cell appearance was seen within 20 min of exposure and seemed most apparent after approximately 1 hr of TPA exposure. Cells treated with TPA for extended periods of time (10 hr or more) had an almost normal appearance.

The transient change in cell morphology was correlated with changes in the levels of oncogene-related RNAs. A single dose of TPA (1 μg/ml) enhanced the level of c-*fos* RNA in all cell lines, irrespective of their life span in vitro. After 50 min of exposure the level of c-*fos* RNA was enhanced at least 20-fold in the mortal, nontumorigenic cell line HU 1752 (Fig. 2). The level of c-*fos* returned to the pretreatment level during the following 2 hr of continued exposure to TPA. A similar transient increase in the c-*fos* level was seen in the immortalized, nontumorigenic cell lines HU 609 and HCV 29. The immortalized, tumorigenic cell lines HCV 29T (Fig. 5) and HU 609T also responded to the TPA treatment by a transient increase (approximately tenfold) in the c-*fos* level. The time course of this induction followed the kinetics observed in the case of the two immortal but nontumorigenic cell lines HU 609 and HCV 29.

The level of c-*myc* increased (at least 20-fold) in the mortal cell line HU 1752 in response to a single dose to TPA (Fig. 2). The increase in the c-*myc* RNA level in this cell line appeared to be transient, like the increase of c-*fos* RNA. However, as has been reported by others [10–12], the decay of the elevated c-*myc* level was slower than the decay in the c-*fos* level (Fig. 2). In contrast to the result obtained in the HU 1752 cell line, we did not observe any increase in the c-*myc* level in the immortalized cell lines. The levels of c-Ha-*ras* and c-Ki-*ras* RNA were unaffected by the TPA treatment in all cell lines studied.

To investigate if the apparent resistance of immortalized cell lines to TPA-enhanced c-*myc* levels was due to the relatively high concentration of TPA used, a dose-response experiment was performed (Fig. 3). The immortalized HCV 29 cell line was exposed to various concentrations of TPA ranging from 5 to 1,000 ng/ml. The exposure time was 45 min. Whereas the level of c-*fos* RNA was positively related

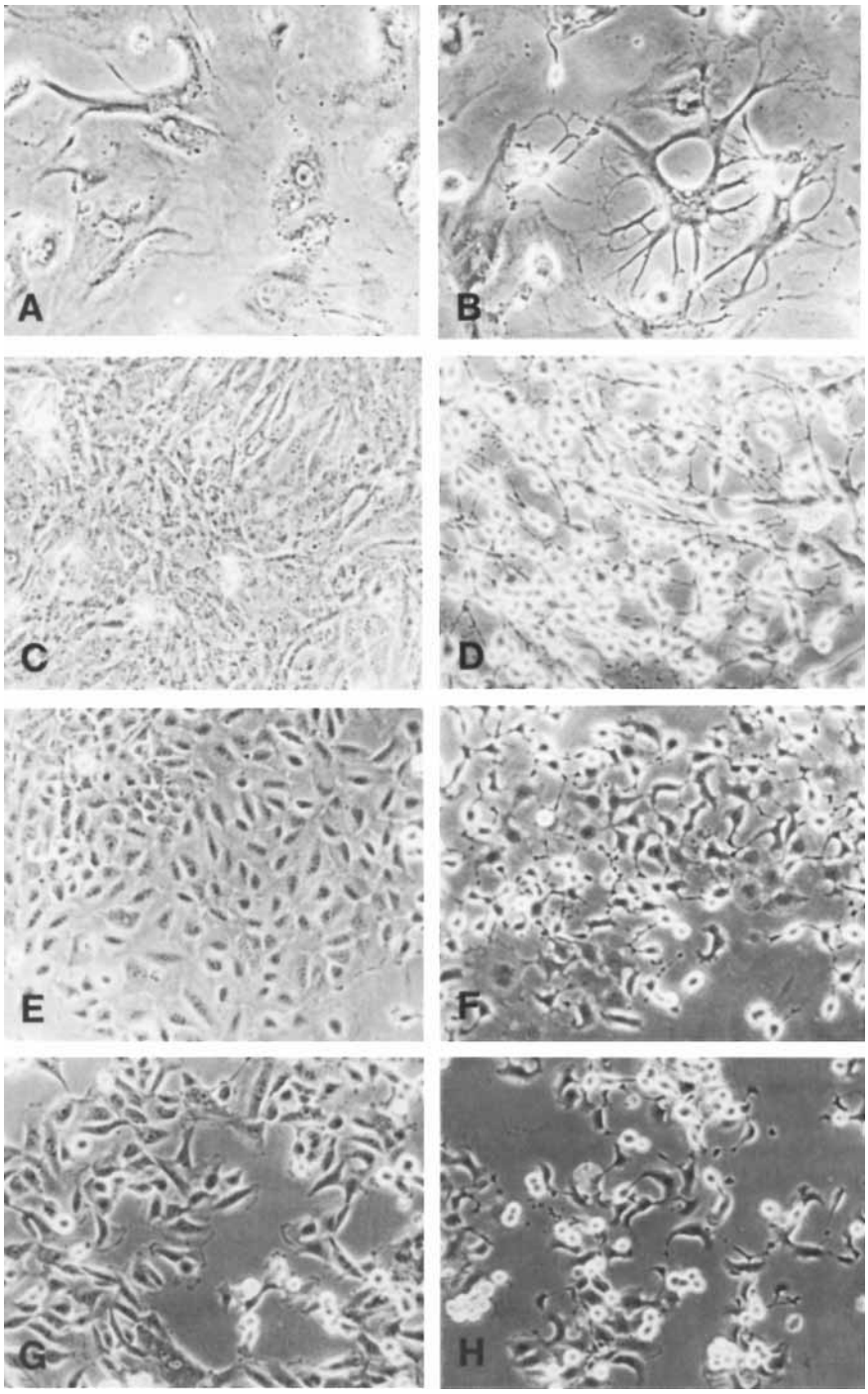

Fig. 1. Effect of TPA (1 μg/ml) on the cell morphology of various human urothelial cells. The mortal HU 1752 cell line before (**A**) and after (**B**) 40 min of exposure. The immortalized HU 609 cell line before (**C**) and after (**D**) 110 min of exposure. The tumorigenic cell lines HCV 29T before (**E**) and after (**F**) exposure for 40 min and HU 609T before (**G**) and after (**H**) 90 min exposure to TPA. ×100.

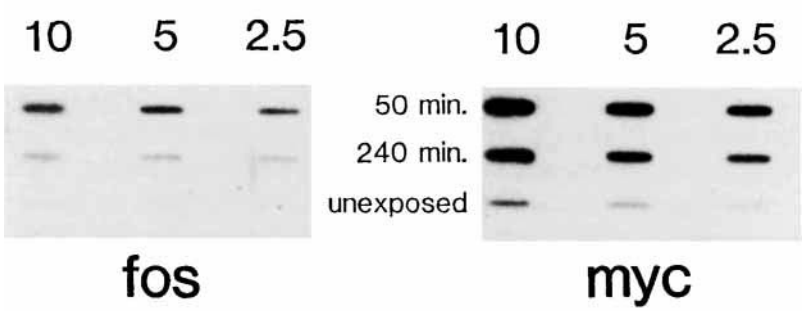

Fig. 2. Slot blot analysis of oncogene expression in the mortal cell line HU 1752 treated with a single dose of TPA (1 μg/ml) in vitro. Cells were lysed and total cellular RNA was extracted after 0, 50, and 240 min exposure, spotted in twofold dilutions (10, 5, and 2.5 μg per slot), and hybridized with radiolabelled DNA probes specific for the *fos* and c-*myc* oncogenes, respectively.

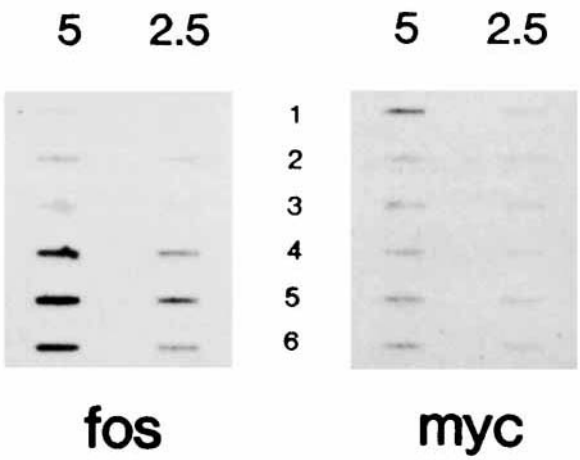

Fig. 3. Slot blot analysis of oncogene expression in the immortalized but nontumorigenic cell line HCV 29. The cells were treated for 45 min with a single dose of TPA: **1**) 5 ng/ml, **2**) 20 ng/ml, **3**) 80 ng/ml, **4**) 200 ng/ml, **5**) 700 ng/ml, and **6**) 1,000 ng/ml. See also legend to Figure 2.

to the TPA concentration, we did not observe any enhancement of the c-*myc*, c-Ha-*ras*, or c-Ki-*ras* RNA levels. The "typical" TPA-induced change in the cell morphology was observed at all concentrations (data not shown).

The basal level of c-*fos* and c-*myc* RNA was measured in all cell lines. The basal level of c-*fos* was essentially the same in all cell lines examined. In contrast, the basal level of c-*myc* RNA varied substantially between cell lines (Fig. 4). The tumorigenic cell lines HCV 29T and HU 609T both showed an enhanced level of c-*myc*. Also the immortalized HU 609 cell line showed an increased basal level of c-*myc* compared to the immortalized HCV 29 and the mortal HU 1752 cell lines.

In order to be able to compare the basal level of oncogene expression in HU 1752 cultured in 20% FBS to the level observed in the other cell lines cultured in 5% FBS, the effect of different serum concentration was examined in HCV 29 (Fig. 4). Neither the basal level of c-*fos* nor c-*myc* was found to be significantly influenced by the serum concentration in the media.

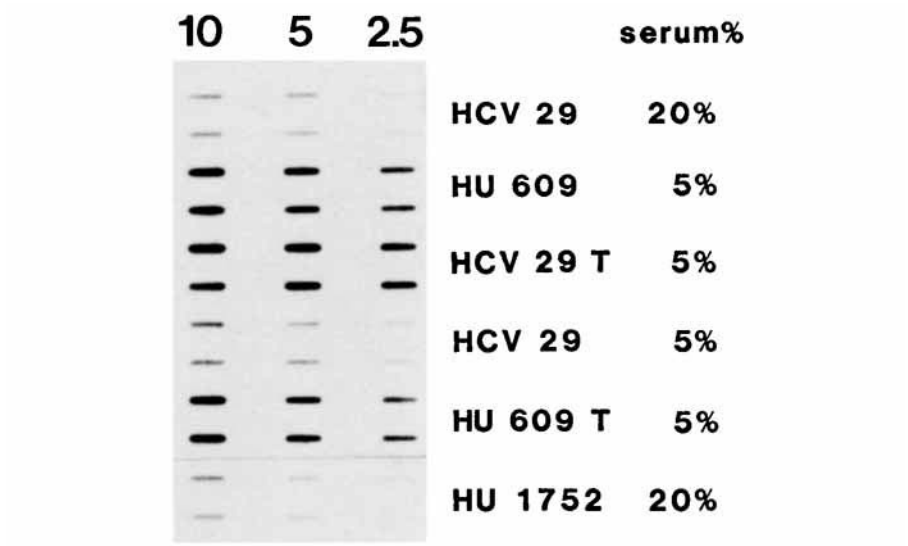

Fig. 4. Basal level of c-*myc* RNA in various human urothelial cell lines. Total cellular RNA was extracted from the indicated cell lines cultured in media containing 5 or 20% fetal bovine serum, respectively. For each cell line RNA was extracted from two independent culture flasks and spotted onto a nitrocellulose filter in twofold dilutions (10, 5, and 2.5 μg). The filter was hybridized with ^{32}P-labelled DNA specific for the human c-*myc*.

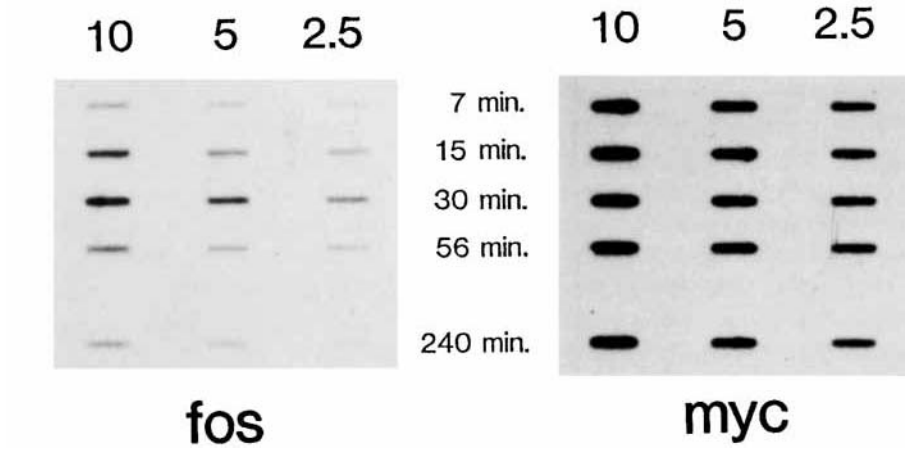

Fig. 5. Slot blot analysis of oncogene expression in the immortal, tumorigenic cell line HCV 29T treated with a single dose of TPA (1 μg/ml) for various times. See also legend to Figure 2.

DISCUSSION

The c-*myc* oncogene has been implicated both in the acquisition of cell immortality and in the ability of cells to form tumors [14–18]. Some information pertaining to this point can be derived by examining the basal level of c-*myc* expression in the cell lines used in this study. The cell lines HU 609, HU 609T, and HCV 29T all show increased levels of c-*myc* RNA, which is consistent with the view that immortalization is related to c-*myc* overexpression. However, the low level of c-*myc* basal expression observed in the immortal line HCV 29 suggests that elevated c-*myc* expression is not always required for immortalization.

Previous work in our laboratory has demonstrated that a single dose of TPA induced expression of the c-*fos* proto-oncogene of HCV 29 cells transiently [13]. This transient induction of c-*fos* correlated with a rapid change in HCV 29 cell morphology. Evidence presented here indicates that TPA transiently induces c-*fos* expression in human urothelial cells irrespective of their life span in vitro and their tumorigenic properties in nude mice. TPA also induced a marked change in cell morphology of all cells investigated. Thus human urothelial cells seem to be susceptible to the action of TPA. Morphological changes have also been observed in other human epithelial cells after TPA treatment—eg, cells of the colon, endometrium, and lung [30–32]. It is interesting to note that the TPA-induced changes in morphology of lung, endometrial, and urothelial cells were transient.

TPA binds to and mediates its cellular effects through the activation of the phospholipid and Ca^{++}-dependent enzyme protein kinase C (pkC) [33–35]; pkC plays a central role in the intracellular signal transduction of a variety of biologically active substances affecting cellular functions and proliferation [34–36]. The enzyme has been found in all tissues so far tested [35], including human urothelial cells [37]. There is ample evidence that substances which activate pkC either directly, like TPA, or indirectly via an enhanced phosphoinositide turnover, like PDGF, transiently activate the transcription of a group of genes named "competence" genes that includes the c-*myc* and c-*fos* proto-oncogenes [10–12,38–43]. Since all urothelial cells judged by the morphology were susceptible to the TPA treatment and since TPA induced at least one of the "competence" genes—namely, c-*fos*—it could be expected that c-*myc* would also be activated by the TPA treatment. Such a coupled induction of both c-*myc* and c-*fos* was, however, observed only in the mortal cell line HU 1752, suggesting a difference in the c-*myc* regulation between immortalized and nonimmortalized human urothelial cells. TPA induces both c-*fos* and c-*myc* expression in the immortalized BALB/c-3T3 [10,43] and immortalized A431 human carcinoma cells [41], thus showing that the immortalization process per se does not cause a decoupling between c-*fos* and c-*myc* in all cell types.

Since the experiments were performed at a high concentration of TPA it was considered possible that the decoupling between c-*fos* and c-*myc* in the immortalized cells was caused by a toxic effect specifically abolishing the activation of c-*myc*. We consider this explanation unlikely because no activation of c-*myc* was observed at low doses of TPA in a dose-response experiment with the immortalized HCV 29 cell line. In this context it is interesting that TPA induced the "characteristic" change in cell morphology at all concentrations investigated. Since c-*fos* only was activated at TPA concentrations higher than 80 ng/ml, the dose-response experiment thus indicates that the morphological change and the c-*fos* activation are two separate responses.

It is probably reasonable to anticipate that cells have a limited capacity for c-*myc* transcription. Thus it is conceivable that activation of pkC may not be able to induce any further c-*myc* expression in cells already expressing maximal levels of this proto-oncogene. Three out of four of the immortalized urothelial cell lines we examined showed an enhanced basal level of c-*myc* which was comparable to the level of c-*myc* expression induced by TPA in HU 1752. It cannot be excluded that c-*myc* expression was maximally expressed in these three cell lines before the TPA treatment. This explanation cannot, however, account for the response of HCV 29 to TPA treatment. This cell line expressed a basal level of c-*myc* RNA that was indistinguishable from the level in the mortal HU 1752, thus illustrating that the

activation of c-*fos* and c-*myc* can also become decoupled in cells expressing low levels of c-*myc*.

Continuous labelling of the urothelial cells with ^{3}H-thymidine showed that 99–100% of the immortalized cells were in the growth fraction, whereas no more than 63% of the mortal HU 1752 cells were in the growth fraction [44]. This indicates that a relative large fraction of the mortal cells (approximately 40%) were at rest at the time of TPA treatment, in contrast to less than 1% of the immortalized cells. We therefore propose that the difference in c-*myc* inducibility between mortal and immortal cell lines reported here is a consequence of the distribution of quiescent vs actively dividing cells. This hypothesis is currently being investigated.

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