

## Altered response to growth factors in rat epithelial liver cells overexpressing human c-Fos protein

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Human *c-fos* cDNA was transfected into normal rat liver epithelial (REL) cells to identify cellular modifications associated with high expression of c-Fos protein. Responses to EGF and TGF $\beta$  were examined in the different cell lines, under anchorage-dependent and -independent conditions. Sensitivity to both factors was modified in transfected cells. While parental cells in monolayer did not respond to EGF, *c-fos* containing cells growth was stimulated by this factor. Overexpression of c-Fos protein led to an enhanced TGF $\beta$ -induced growth inhibition under anchorage dependent conditions, and TGF $\beta$  abolished spontaneous growth in soft agar of the cell lines containing *c-fos* oncogene. The mechanisms underlying the increased sensitivity to TGF $\beta$  in *c-fos* transfected cells are still to be determined.

*c-fos*; TGF $\beta$ ; EGF; Rat liver epithelial cell

### 1. INTRODUCTION

Expression of *c-fos* is observed at low or undetectable levels in most exponentially growing cells although it can be activated transiently in response to numerous stimuli including growth and serum factors (for review see [1]). The *c-fos* gene seems to be involved in the regulation of the normal cell cycle [2] and differentiation [3]. This gene encodes a labile 55 kDa phosphorylated protein localized in the cell nucleus and likely acting as a transcriptional regulator in association with the members of the *jun* family in the AP1 complex [4].

To investigate the intervention of c-Fos protein in phenotypic transformation and identify the biological modifications associated with a high expression of the onco-protein, we transfected human *c-fos* cDNA into a clone of rat liver epithelial (REL) cells.

Responsiveness to growth factors was examined in the different cell lines. We chose to focus on two growth factors: epidermal growth factor (EGF) and transforming growth factor  $\beta$  (TGF $\beta$ ). EGF, mitogenic for most cell types, has been shown to display complex effect in REL cell cultures [5]. TGF $\beta$  has been found to be a potent inhibitor of the in vitro growth of a variety of epithelial cells including mammary epithelial cells [6], liver epithelial cells [7] and keratinocytes [8]. The TGF $\beta$  group includes at least three highly homologous genes (TGF $\beta$ 1, TGF $\beta$ 2, TGF $\beta$ 3) present in humans and other mammals, with additional genes present in avian and amphibian genomes [9,10]. All TGF $\beta$  forms tested display inhibitory activity in epithelial cell proliferation

and mesenchymal differentiation but the various isoforms of TGF $\beta$  are multifunctional and their primary functions are not yet known [11]. However, the fact that most cells examined possess functionally active, high affinity cell surface receptors for TGF $\beta$  implies a fundamental role for this protein in normal cell physiology [11]. TGF $\beta$  has raised increasing interest recently, as it has been suggested that acquisition of resistance to growth inhibitors could be a primary event in carcinogenesis [12].

The present report describes the modifications noted in the *c-fos* containing cell lines.

### 2. MATERIALS AND METHODS

#### 2.1. Materials

EGF and TGF $\beta$ 1 were obtained from Sigma (France). Restriction enzymes were from Boehringer-Mannheim (Meylan, France).

#### 2.2. Cell culture and oncogene transfection

The rat liver epithelial (REL) cell culture used in these studies was established from normal rat liver, as described previously [13,14]. REL cells were grown in Ham's F10 medium containing 10% fetal calf serum (FCS) and antibiotics at 37°C in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub>. Because Lin et al. [7] have shown that sensitivity of RELC to TGF $\beta$  decreases with increasing passage of RELC in culture, all cell lines were tested for TGF $\beta$  responsiveness at similar passage number and up until 28th passage. Low-passage of rat liver epithelial cells (REL) were transfected by using the calcium phosphate precipitate method [15] with the pM43.1 plasmid [16], a generous gift from M. Piechaczyk (URA CNRS 1191 - France). This plasmid was constructed by cloning a nearly full-length human *c-fos* cDNA into the *Bam*HI site of the pZIP neo SV(X) retroviral vector [17]. Neomycin-resistant clones were selected in the presence of 600  $\mu$ g/ml of G418 (Gibco-BRL, France).

#### 2.3. Southern blot analysis

High-molecular-weight nuclear DNA from REL cells was isolated

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as described by [18]. DNA (10  $\mu$ g) was incubated with 50 units of *Eco*RI for 20 h at 37°C. The digested DNAs were electrophoresed through a 1% agarose gel and transferred to a nylon filter (Amersham). Hybridization with the 940 bp *Bam*HI-*Apal* restriction fragment of the human *c-fos* cDNA radiolabeled by the multiprime DNA labeling system (Amersham) was carried out at 42°C as described by [19]. The specific activity of this probe was approximately  $1 \times 10^9$  cpm/ $\mu$ g. After a 20 h period of hybridization, filters were washed three times in  $2 \times$  SSPE ( $1 \times$  SSPE is 0.18 M NaCl, 20 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM EDTA pH 7.4) at room temperature for 5 min and once successively in  $1 \times$  SSPE,  $0.5 \times$  SSPE,  $0.1 \times$  SSPE at 65°C for 30 min. Autoradiography was carried out at -80°C with intensifying screens (Philips Universal).

#### 2.4. Northern blot analysis

Total cellular RNA was extracted from confluent cultures by the guanidine isothiocyanate procedure [20] followed by centrifugation over CsCl gradients [21]. For Northern blot analysis, RNA species (25  $\mu$ g) were separated on a 1% agarose gel containing 2.2 M formaldehyde and then transferred to Hybond nylon membranes (Amersham) for hybridization with  $^{32}$ P-labeled *Bam*HI-*Apal* restriction fragment of human *c-fos* cDNA. Hybridization and washing were carried out as described above.

#### 2.5. Western blot analysis

Nuclear extracts from transfected, parental or control REL cells were prepared essentially as described by [22]. Western blotting and immunodetection of Fos were carried out according to [23]. The highly specific anti-Fos antibody, used in this study, was a kind gift from Dr. B. Verrier (U103 CNRS-BioMerieux, France).

#### 2.6. Cell growth experiments and soft agar assays

Cells were plated at 70 000 cells/35 mm culture dish (Falcon plastics) in Ham's F10 supplemented with 10% FCS and allowed to attach overnight. Following attachment, cultures were washed twice and incubated overnight in serum-free medium. Then, media were replaced with serum supplemented medium containing various concentrations of TGF $\beta$ . The inhibition of growth by TGF $\beta$  was assessed by cell counting using a coulter cell counter.

For the soft agar assays, one ml of cell suspension ( $5 \times 10^3$ /ml) in 0.28% agar (FMC Bioproducts, USA) in Ham's F10 containing 5% FCS and additional growth factors (EGF: 7 ng/ml and TGF $\beta$ : 2 ng/ml)

were pipetted onto a 2 ml base layer (containing 0.4% agar in Ham's F10 supplemented with FCS 5%) in 35 mm Petri dishes. Plates were incubated at 37°C for 14 days and colonies larger than 50  $\mu$ m were scored as positive.

### 3. RESULTS

After transfection of REL cells with the plasmid pM43.1 carrying the human *c-fos* cDNA and G418-resistance gene, and selection with G418, three surviving colonies were obtained. These colonies were isolated and characterized. Because they presented similar characteristics, only the 43C clone will be described in this paper. No G418-resistant clones arose from untransfected REL cells, and the control REL cells, transfected with the plasmid pZIP neo SV(X) containing the G418 resistance gene alone [17], was not affected in morphology and growth.

The integration of the *c-fos* oncogene in the *c-fos* transfected REL cells (43C) was demonstrated by Southern blot analysis (Fig. 1A). The 23 kb, 12 kb and 9 kb *Eco*RI restriction fragments observed in transfectant were absent from parental cell DNA. The constitutive expression of the pM43.1 derived mRNA was detected by Northern blot in 43C only (Fig. 1B). Finally, the integrity and the nuclear localization of the c-Fos protein was confirmed by Western blot analysis (Fig. 1C). The pattern obtained with 43C nuclear extract was similar to the one obtained from serum stimulated Ltk<sup>-</sup> cells, used as control [16].

The morphology of 43C was unchanged, compared to control and parental REL cells. Nevertheless, cell proliferation was markedly increased in 43C cells and this line formed colonies in soft agar in the presence of 5% FCS alone (Fig. 3).

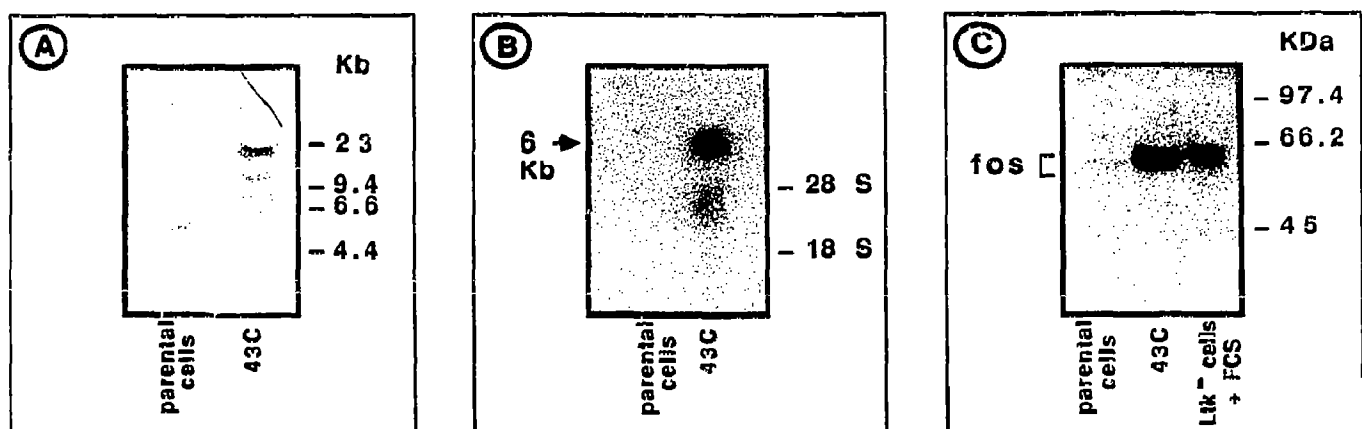


Fig. 1. Southern blotting analysis of *c-fos* gene (A), expression of pM43.1 RNA (B) and c-Fos protein (C). A. DNAs (20  $\mu$ g) extracted from parental and *c-fos* transfected cells were digested by *Eco*RI. Lambda phage DNA *Hind*III and *Phi*X DNA *Hae*III generated fragments were used as molecular weight markers in kilobases. B. Expression of pM43.1 RNA in 43C line was revealed with  $^{32}$ P-labeled human fragment of *c-fos* cDNA (see [16]). Positions of 28S and 18S rRNAs are shown and equal amounts of total RNAs were loaded on filter when stained with ethidium bromide. Arrow indicates pM43.1 mRNA. C. Expression of human c-Fos protein in 43C is similar to the pattern of c-Fos protein expression in serum-stimulated Ltk<sup>-</sup> cells used here as control. No c-Fos expression is detectable in parental cells. The positions of prestained molecular size markers are shown on the right.

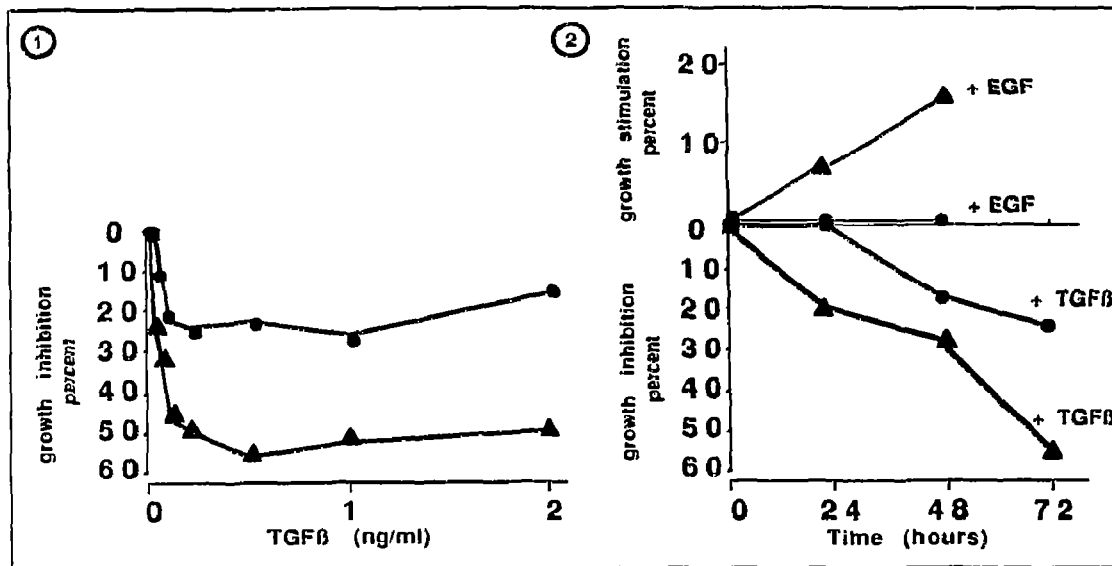


Fig. 2. Monolayer growth. (1) TGFβ dose-response: growth inhibition percentage of parental cells (●) and 43C cells (▲) in presence of different concentrations of TGFβ (0.02/0.05/0.1/0.2/0.5/1/2 ng/ml). (2) EGF and TGFβ kinetic curves. (●) parental cells, (▲) 43C cells. Each assay point was performed in triplicate in which the values differed by no more than 3.4%.

Parental and transfected REL cells were examined for responsiveness to growth inhibition induced by TGFβ1. Fig. 2.1 and Fig. 2.2 illustrate representative TGFβ dose-response and kinetic curves under anchorage-dependent conditions and Fig. 3 summarizes the effects of TGFβ treatment on colony formation in soft agar assay. Treatment with TGFβ of parental REL cells

caused a dose-dependent inhibition of cell proliferation which reached a maximum of 30%. The *c-fos* containing cell line (43C) seemed twice as sensitive to the TGFβ than the parental cells. Indeed, the inhibition observed in 43C cell cultures was higher (57%) in monolayer growth (Fig. 2.1) and arose with a shorter latency period in 43C cells (24 h) than in parental REL cells (48

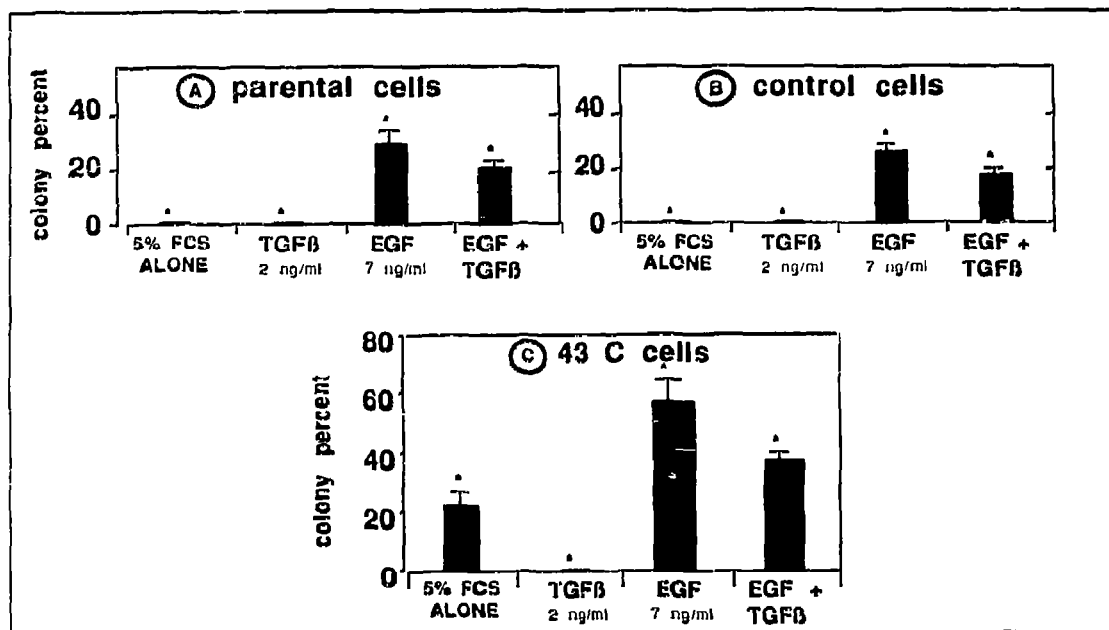


Fig. 3. Anchorage independent growth in soft agar of parental cells (not transfected cells), control cells (transfected with a plasmid containing the G418 resistance gene alone) and 43C cells (*c-fos* transfected cells) in presence of growth factors at the indicated concentration. Each value is the mean of triplicate dishes.

h) (Fig. 2.2). Moreover, the colony formation in soft agar was completely abolished in *c-fos* transfected line by 2 ng/ml of TGF $\beta$  (Fig. 3).

Tsao et al. have shown that the in vitro proliferation of normal REL cells may be either inhibited or stimulated by EGF depending on the passage number, and that the effect of growth modulators on the proliferation of REL cells may be altered by the extracellular substrate on or in which they grow [5]. Therefore, we also studied the effect of EGF (with or without TGF $\beta$ ) on cell growth in monolayer and in soft agar. While anchorage-dependent growth was increased only in 43C cells by EGF and remained unchanged in control or parental cells (Fig. 2), in soft agar assay, all cell lines were stimulated by EGF and presented significant colony formation (Fig. 3).

TGF $\beta$  at 2 ng/ml inhibited the EGF-induced colony formation in all the REL cell lines.

#### 4. DISCUSSION

We have established a rat epithelial liver cell line (43C line) producing a constitutive expression of protein encoded by the human *c-fos* cDNA. The cDNA was transcribed into a single mRNA species of 6 kb which corresponds to the genomic pM43.1 RNA [16]. The c-Fos protein was detected in the nucleus and the pattern obtained with 43C was similar to that of serum stimulated Ltk<sup>-</sup> cells used as control [16].

The proliferation of *c-fos* transfected cells (43C) was increased comparatively to the parental REL cells and, in the presence of EGF, only the 43C cells exhibited an enhanced growth rate. It has been shown that proliferation of early passage of REL cells in anchorage-dependent conditions was inhibited by EGF [5]. Moreover, it was demonstrated that these cell lines contain a heterogeneous population of cells which responded differently to EGF and in particular, a small fraction of this heterogeneous population resisted this effect and was stimulated by this treatment [5]. In our study, the proliferation of the parental line was not affected by EGF. This may be due to the fact that the parental cell line was a clonally derived cell-strain, and therefore a homogeneous population in which the phenotype of EGF-induced growth inhibition was diminished. In opposite, the 43C cell line transfected with *c-fos* was sensitized to anchorage-dependent growth stimulation by EGF. Thus, cells that contain an activated *c-fos* oncogene seemed to exhibit an increased sensitivity to the growth-promoting activity of normal growth factor, EGF, and respond to it in a qualitatively different manner than the parental cells.

In contrast, in soft agar culture, parental and *c-fos* transfected REL cell lines presented colony formation in presence of EGF. Whereas *c-fos* containing cell growth was stimulated by EGF regardless of the an-

chorage dependency, control or parental cells responded differently to EGF, depending on the culture conditions. EGF action on REL cell cultures is complex and highly variable. Similar findings with retinoic acid have also been reported in rat epithelial cell lines [24].

It has been suggested that acquisition of resistance to normal growth inhibitors might well be the primary event in carcinogenesis or at least a necessary event occurring later in the process [12]. Indeed, although anchorage dependent growth of most normal epithelial cells was inhibited by TGF $\beta$ , many transformed epithelial cell lines were resistant to this inhibitory effect [25,26].

Our results demonstrated that the introduction of a human *c-fos* cDNA into REL cells led to an increased sensitivity to the growth inhibitory effects of TGF $\beta$  under both anchorage dependent and independent conditions. On the other hand, the results of the soft agar assay clearly showed that transfected cells were partially transformed since they showed significant colony formation in the presence of 5% FCS [27]. Many studies have reported a resistance to TGF $\beta$  in transformed cells [26], but our results are not necessarily inconsistent with the theory that resistance to growth inhibitory factor is an important and/or obligate event during carcinogenesis [12]. c-Fos protein is known to be a pleiotropic protein acting as both a transrepressor and a transactivator of transcription [28]. In light of our data, one could speculate on a direct action of the oncoprotein on the synthesis of TGF $\beta$  or its receptor, or on a postreceptor mechanism intensifying the response to TGF $\beta$ . Further work needs to be done to determine the mechanisms underlying the increased sensitivity to this growth factor in *c-fos* transfected REL cells.

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