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Analysis of the differentiation-promoting potential of inducible c-*fos* genes introduced into embryonal carcinoma cells

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To investigate the differentiation-promoting potential of c-*fos* in embryonal carcinoma cells (EC cells) we have designed various human metallothionein promoter-mouse-c-*fos* gene constructs containing also the selectable SV40 promoter-driven *neo* gene. Upon transfection into F9 EC cells and selection for *neo* resistance, the following results were obtained. (i) With each of the constructs, colonies of morphologically altered and differentiated (i.e., TROMA-1 and TROMA-3 expressing) cells were identified. (ii) Expression of c-*fos* was required to affect the differentiation state of F9 cells to a significant extent, but a low level was sufficient; no enhancement of differentiation was noticeable even after 100-fold induction of c-*fos* expression by cadmium. (iii) F9 cell clones were isolated which, in spite of very high levels of exogenous c-*fos* expression, had stem cell morphology. These cells, however, continuously generated morphologically altered and differentiated cells upon subculturing. (iv) In other EC cell lines, which resemble stem cells more closely than the 'partially differentiated' F9 cells, c-*fos* expression showed either a less pronounced (P19 cells) or no differentiation-promoting effect at all (PC13 cells). Our results suggest that the c-*fos* gene product acts in concert with other, probably 'spontaneously' occurring events to promote differentiation of certain EC cell lines.

Key words: differentiation/embryonal carcinoma cells/metallothionein promoter/gene expression/proto-oncogene

Introduction

Recent studies have implicated the products of two proto-oncogenes in cellular differentiation processes. The cellular homolog c-*src* of the Rous sarcoma virus oncogene is expressed at high levels specifically during organogenesis of neural tissues in both chickens and human (Cotton and Brugge, 1983; Jacobs and Ruebsamen, 1983; Levy *et al.*, 1984; Sorge *et al.*, 1984; Fults *et al.*, 1985). In both chick neural retina and cerebellum, c-*src* expression occurs in developing neurons at the onset of differentiation at a stage when cell proliferation ceases (Sorge *et al.*, 1984; Fults *et al.*, 1985). Another proto-oncogene-encoded product implicated in differentiation processes is the c-*fos* protein, as suggested by its highly tissue- and stage-specific expression. High levels of c-*fos* mRNA and protein are detectable specifically in the fetal membranes (Müller *et al.*, 1983; Curran *et al.*, 1984) and in certain hematopoietic cells (Müller *et al.*, 1984a). In the fetal membranes, c-*fos* mRNA levels increase as gestation proceeds (Müller *et al.*, 1983), and in cells of the myelomonocytic lineage c-*fos* expression is restricted to differentiated macrophages (Gonda and Metcalf, 1984; Müller *et al.*, 1984a,1985; Mitchell *et al.*, 1985). Expression of normal exogenous c-*fos* genes has been reported to affect the differentiation state of 'nullipotent' F9 embryonal carcinoma cells (EC cells) (Müller and Wagner, 1984), providing direct evidence for a differentiation-promoting potential of c-*fos*.

The main goal of the present study was to analyze whether c-*fos* protein expression in F9 EC cells is sufficient for the induction and completion of such a complex process as cellular differentiation. We also intended to investigate which level of c-*fos* expression is required (or sufficient) to affect cellular differentiation. Finally, we addressed the question as to whether other EC cell lines respond to expression of exogenous c-*fos* genes in a way similar to F9 cells, or whether the observed biological effect is F9 specific.

Results

Inducible c-fos gene constructs

To analyze how the expression of exogenous c-*fos* genes correlates with the induction of differentiation in EC cells, c-*fos* gene constructs were designed so as to meet at least three requirements: (i) the different constructs should give rise to different levels of c-*fos* expression; (ii) transcription should be inducible; and (iii) the constructs should contain a selectable marker gene. The basic construct (p19/1; Figure 1) contains the c-*fos* gene under the control of the cadmium-inducible human metallothionein promoter (Karin *et al.*, 1984) and the bacterial *neo* gene with the SV40 promoter (pSV2-neo; Southern and Berg, 1982) conferring resistance to the antibiotic G418 in eukaryotic cells. Translation start and stop codons as well as the polyadenylation signal are the natural c-*fos* sequences. This construct should therefore direct the synthesis of normal c-*fos* protein. To obtain higher levels of c-*fos* expression two manipulations were carried out: deletion of sequences in the 3'-non-translated region of c-*fos*, which have previously been shown to inhibit expression of c-*fos* mRNA or

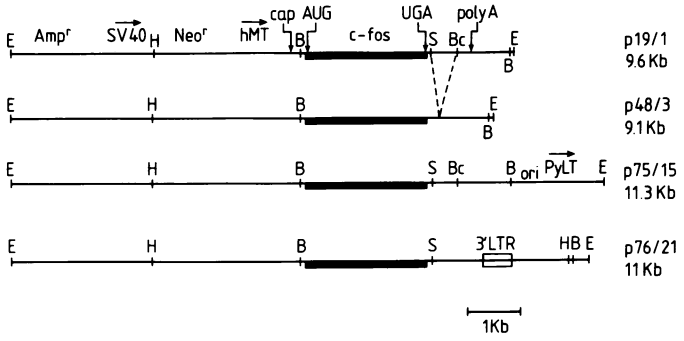

Fig. 1. Inducible mouse c-*fos* gene constructs. For details of the construction, see Materials and methods. E, *Eco*RI; B, *Bam*HI; Bc, *Bcl*I; H, *Hind*III; S, *Sal*I; Amp^r, ampicillin resistance gene; SV40, SV40 enhancer and promoter; Neo, kanamycin as well as G418 resistance gene; hMT, human metallothionein IIA promoter; cap, initiation site of transcription; AUG, start codon for translation; UGA, stop codon for translation; polyA, poly(A) addition signal; oriPyLT, polyoma origin of replication and enhancer, promoter and 5' 40% of the sequence coding for large T antigen; LTR, long terminal repeat of the proviral FBJ-MuSV.

Table I. Transfection of c-*fos* gene constructs on F9 and P19 EC cells: quantitative evaluation

Construct	Number of G418^r-colonies/ μg plasmid	% Morphologically altered colonies
Recipient cells: F9 EC cells		
pSV-neo	20	<1%[a]
p19/1	60	~10%
p48/3	40	~10%
p75/15	150	~50%
p76/21	100	~20%
Recipient cells: P19EC cells		
p19/1	70	<1%[a]
p48/3	40	<1%[a]
p75/15	100	~10%

[a]No morphologically altered colonies detectable.

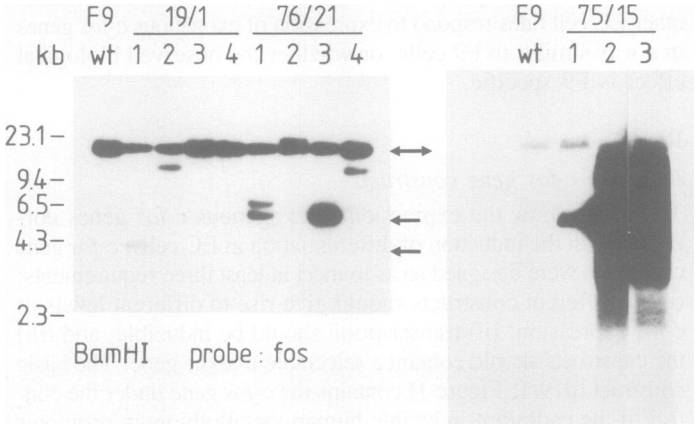

Fig. 2. Analysis of individual clones for exogenous c-*fos* genes. DNA was digested with *Bam*HI and analysed by the Southern blot technique. F9 wt are untransfected control cells. The arrows show the expected position of an intact exogenous c-*fos* gene. The double arrow shows the position of the endogenous c-*fos* gene. Migration of *Hind*III-digested λ phage DNA (marker) is indicated.

to decrease its stability (Miller *et al.*, 1984) (construct p48/3; Figure 1); and insertion of a long terminal repeat from FBJ murine osteosarcoma virus (FBJ-MuSV) 3′ from the coding region (construct p76/21; Figure 1).

To test whether it might be possible to obtain F9 cells that continue proliferation in a differentiated state, another construct, p75/15 (Figure 1), was derived from p19/1 by introducing part of the polyoma virus early region 3′ to the polyadenylation site of c-*fos*. The inserted polyoma sequences included the origin of replication, the promoter, the enhancer and the amino-terminal 40% of the large T antigen coding region, which have been shown to be sufficient for immortalization of murine fibroblasts (pLT214; Rassoulzadegan *et al.*, 1983).

Transfer of inducible c-fos gene constructs into F9 stem cells

The different DNA constructs were introduced into F9 stem cells by calcium phosphate co-precipitation followed by selection in G418-containing medium. With each of the four constructs, significant numbers of morphologically altered F9 cells were observed. Ten to 50% of all colonies exhibited a morphologically altered phenotype, the highest number of such colonies obtained with construct p75/15 (Table I). Generally, three types of colonies were observed: colonies consisting of either morphologically normal stem cells or of morphologically altered cells only (as

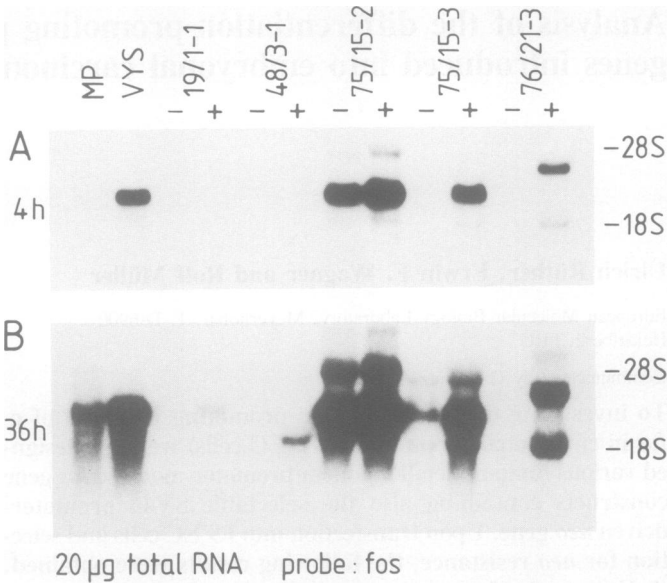

Fig. 3. Expression and inducibility of exogenous c-*fos* genes. RNA from clones grown in the absence or presence of cadmium (5×10^{-6} M; 6–8 h) was separated on formaldehyde-agarose gels, blotted onto nitrocellulose paper and hybridized to the mouse c-*fos* probe. MP, RNA from adult mouse macrophages; VYS, RNA from day 18 visceral yolk sac. Two exposures (4 h, **panel A,** and 36 h, **panel B**) of the same filter are shown. The migration of 18S/28S rRNAs is indicated.

previously reported, Müller and Wagner, 1984), and colonies that were comprised of both morphologically normal stem cells (mostly in the center of the clone) and morphologically altered cells at the edges. The number of morphologically altered colonies obtained with pSV-neo alone was practically zero, indicating that the G418 selection procedure does not select for 'partially differentiated' cells present in the starting population.

Genomic organization of exogenous c-fos genes in individual clones

Individual clones established by transfection with each of the four constructs were analyzed for the presence, organization, expression and inducibility of exogenous c-*fos* sequences. The detection of intact exogenous c-*fos* genes was facilitated by the presence of a new *Bam*HI site introduced during construction of the recombinant plasmids (see Materials and methods). As shown by Southern blot analysis (Figure 2), most clones analyzed were found to contain exogneous c-*fos* genes (clones 19/1-1; 76/21-1; 76/21-3; 75/15-1; 75/15-2; 75/15-3). In some instances a band of different size from that expected was observed, presumably due to loss of one of the two *Bam*HI sites in the exogenous DNA after transfection (e.g., clone 19/1-2). In a few clones, no exogenous c-*fos* genes were detectable (e.g., clones 19/1-3; 19/1-4). With constructs p19/1, p48/3 and p76/21 only one or a few copies (maximum 10) were found per diploid genome. In contrast, plasmid p75/15 was present as >100 copies per cell in three out of four clones. These sequences were arranged in tandem repeats (data not shown). Amplification of p75/15 and p76/21 constructs upon transfection of F9 EC cells may also be responsible for the higher number of G418^r colonies relative to constructs p19/1 and p48/3 (Table I).

Expression and inducibility of exogenous c-fos genes in individual clones

The level of c-*fos* mRNA expression after induction by cadmium (6–8 h; 5×10^{-6} M) was found to correlate with the number of exogenous c-*fos* gene copies (Figures 2,3; Table II). The fac-

Table II. Analysis of individual clones with stem cell morphology isolated after transfection of F9 EC cells with c-*fos* gene constructs

Construct	Cell clone	Exogenous c-*fos* DNA copies/cell[a]	Rel. number of mRNA copies without induction[a]	Rel. number of mRNA copies after induction with cadmium[a]	Factor of induction[a]	Presence of differentiated cells[b]
p19/1	19/1-1	2	1	4	4	+
	-2	1	2	2	1	+
	-3	0	–	–	–	–
	-4	0	–	–	–	–
p48/3	48/3-1	8	1	18	18	+
	-3	1	2	2	1	+
	-4	1	0	0	–	–
p75/15	75/15-1	21	2	20	10	+
	-2	>100	>100	>100	2	+
	-3	>100	~20	>100	~45	+
	-4	>100	~20	>100	~45	+
p76/21	76/21-1	3	2	20	10	+
	-2	1	1	25	20	+
	-3	29	1	>100	>100	+
	-4	1	0	0	–	–

[a]As determined by density scanning of the blot autoradiograms.
[b]Presence of morphologically altered cells (e.g., Figure 2B,C) and cells expressing the differentiation marker TROMA-1 (e.g., Figure 6A-C). +, >1% TROMA-1-positive cells; –, <0.01% TROMA-1-positive cells, i.e., similar to F9 EC cells.

tor of induction, however, did not follow a strict rule. With each of the constructs, clones were obtained showing cadmium-regulated as well as constitutive expression. The highest levels of c-*fos* mRNA and the greatest factors of induction were observed in clones established after transfection with constructs p75/15 and p76/21 (Table II). In clones 75/15-2, 75/15-3 and 76/21-3 the concentration of c-*fos* mRNA was close to that in late-gestation visceral yolk sac, where c-*fos* expression occurs naturally at a very high level (Müller *et al.*, 1983). In clones 19/1-1 and 48/3-1, in the absence of cadmium, the level of c-*fos* mRNA was relatively low, i.e., ~1/20 of that in mouse macrophages (Müller *et al.*, 1984a).

Evidence that c-fos expression is not sufficient to affect the differentiation state of F9EC cells

When stem cell type clones were isolated and the cells replated, a certain fraction of morphologically altered cells was detectable with all clones showing expression of exogenous c-*fos* genes (Figure 3; Table II). This fraction of morphologically altered cells remained more or less the same even after many passages, indicating that these cells could not have originated from the primary colony. Such cells, due to their inability to proliferate (this was also found with cell lines expressing polyoma large T antigen from construct p75/15; data not shown), would have been rapidly overgrown by stem cells. The morphologically altered F9 cells therefore must have arisen in a continuous process from proliferating, morphologically normal stem cells. These findings were the first indication that c-*fos* expression may not be the sole requirement for the induction of morphological alterations in F9 cells. This notion is in agreement with the observation that the fraction of morphologically altered cells could not be increased by cadmium treatment for 8 h (longer exposure times led to lethal effects), and subsequent incubation in normal medium for at least 3 days.

Although c-*fos* expression was clearly required to affect the differentiation state of F9 cells to a significant extent (Table II), the level of c-*fos* expression did not show any correlation with the fraction of morphologically altered cells in a given clone. Thus, clones 19/1-2 and 76/21-3 with very low levels of c-*fos* expression showed almost 100% morphologically altered cells, whereas cultures of clone 75/15-2 contained predominantly stem cell type colonies, in spite of the extremely high level of c-*fos* expression (Figures 3,4; Table II). These observations suggest that an increase of c-*fos* expression may not necessarily lead to a higher number of morphologically altered cells.

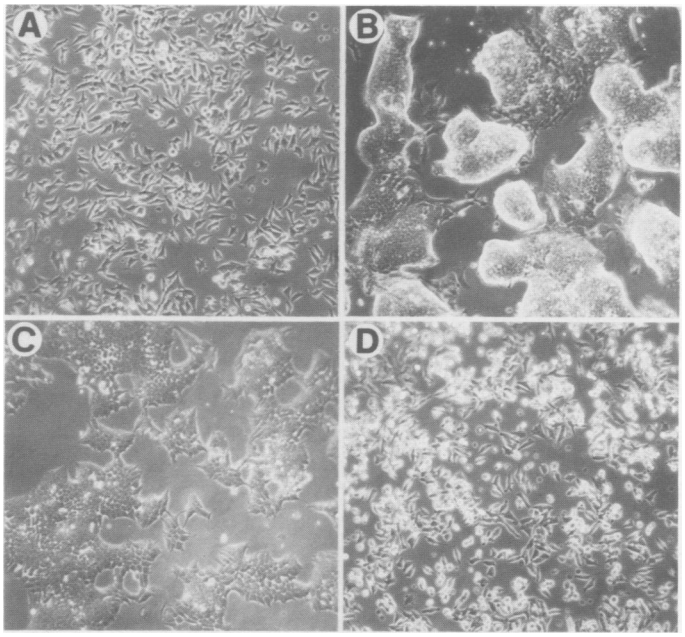

Fig. 4. Morphology of F9 clones carrying different c-*fos* gene constructs. **A,** clone 19/1-2; **B,** clone 48/3-3; **C,** clone 75/15-2; **D,** clone 76/21-3. G418^r morphologically normal clones were isolated ~14 days after transfection and reseeded. The dense colonies in **panel B** have typical F9 stem cell morphology, whereas most cells in **panels A** and **D** are morphologically different from stem cells. Phase contrast pictures, photographic magnification 160x.

Synthesis of c-fos protein

To analyze the size and level of c-*fos* protein in F9 cells transfected with construct p75/15, cells were metabolically labeled with [^{35}S]methionine and c-*fos* protein was immunoprecipitated with a specific tumor-bearing rat (TBR) serum (Curran and Teich, 1982a). This antiserum detects *fos* protein of 55 kd relative mol.

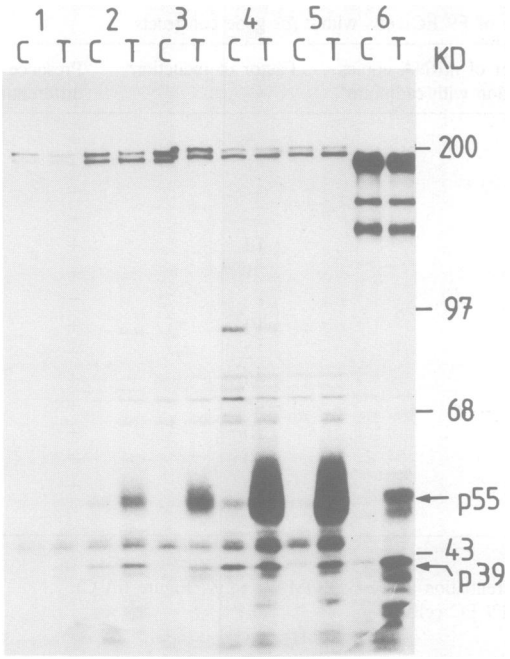

Fig. 5. Immunoprecipitation of [^{35}S]methionine-labeled protein with *fos*-specific antibodies. For experimental details, see Materials and methods. **1,** normal F9 stem cells; **2,** clone 75/15-3 in the absence in cadmium; **3,** clone 75/15-3 induced with cadmium (5×10^{-6} M; 5 h); **4,** clone 75/15-2 in the absence of cadmium; **5,** clone 75/15-3 in the presence of cadmium; **6,** RS2 cells. C, control (normal rat) serum; T, *fos*-specific TBR serum. The migration of marker proteins is indicated. p55, position of unmodified *fos* protein; p39, cellular protein completed with *fos*.

wt. (p55) in the nucleus of FBJ-MuSV-transformed cells (Curran and Teich, 1982a). Normal cells, such as amnion cells, macrophages and growth factor-stimulated fibroblasts contain, in addition, modified c-*fos* protein of higher mol. wt. (p57-62) (Curran *et al.*, 1984; Müller *et al.*, 1984a,b, 1985; Kruijer *et al.*, 1984; Mitchell *et al.*, 1985). Both, cellular and viral gene products are complexed with a cellular protein, p39 (Curran and Teich, 1982b; Curran *et al.*, 1984, 1985). Figure 5 shows that the levels of labeled c-*fos* protein in clones 75/15-2 and 75/15-3 correlated with the level of c-*fos* mRNA. Both, c-*fos* protein of normal size (55 kd and higher mol. wt. forms of up to 60 kd) and p39 were detectable in these clones. The level of labeled c-*fos* protein in clone 75/15-3 cells was similar to that in FBJ-MuSV-transformed 208F fibroblasts (RS2 cells; Curran and Teich, 1982a), and increased after induction with cadmium. Extremely high levels of labeled c-*fos* protein (>20-fold more than in RS2 cells) were observed in clone 75/15-2. Further analyses of [^{32}P]orthophosphate-labeled cells showed that the c-*fos* gene products in clones 75/15-2 and 75/15-3 are phosphoproteins (data not shown).

Expression of TROMA-1 and TROMA-3 intermediate filament proteins in individual cells

Differentiation of F9 stem cells has previously been reported to be accompanied by the expression of intermediate filaments, recognized by monoclonal antibodies TROMA-1 and TROMA-3 (Boller and Kemler, 1983; Müller and Wagner, 1984). When the clones established in this study were analyzed for TROMA-1 expression, generally four types of colonies were observed with each of the clones analyzed (Figure 6, A–C shows TROMA-1 expression in clone 75/15-2): (i) colonies showing no TROMA-1

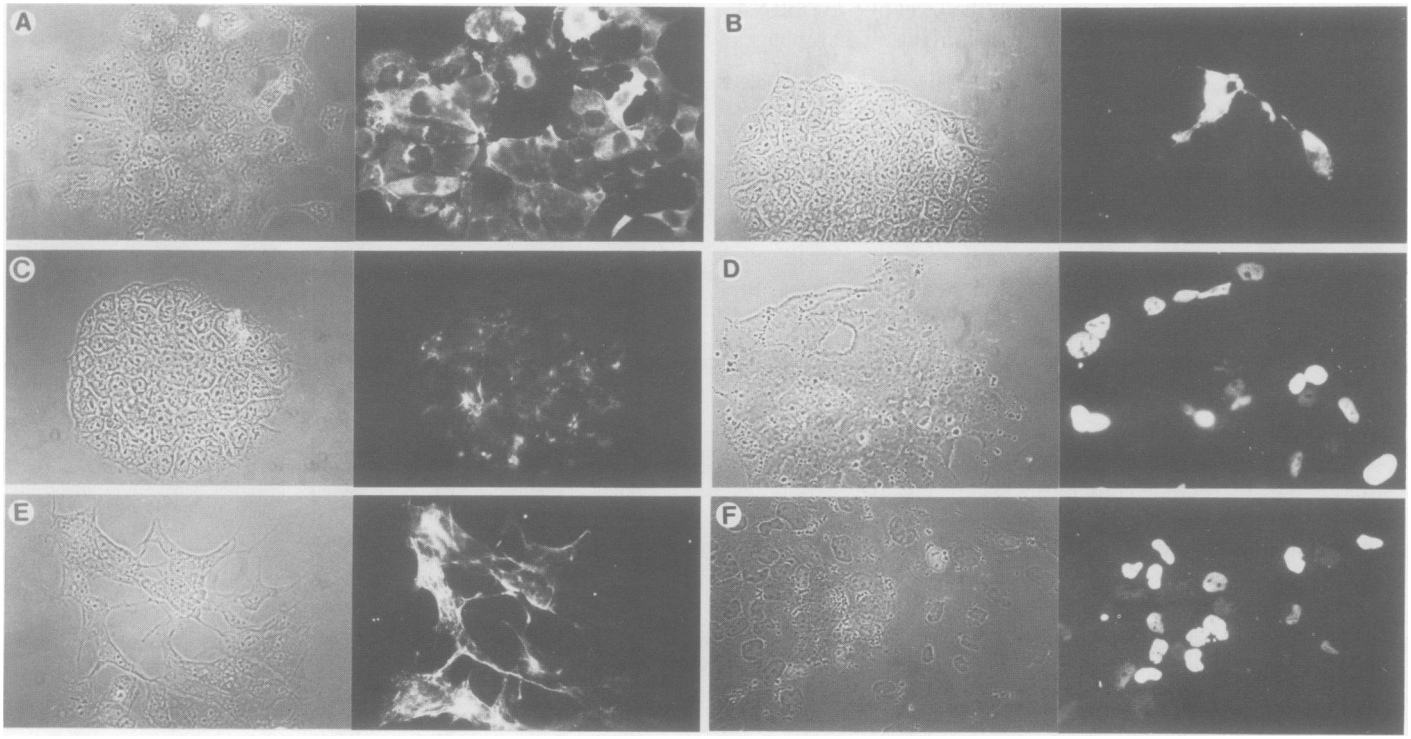

Fig. 6. Expression of TROMA-1 and c-*fos* in individual cells of clone 75/15-2. Cells were fixed and stained by double-antibody immunofluorescence as described in Materials and methods. **A, B, C:** expression of TROMA-1 in clone 75/15-2 cells; **D,** expression of c-*fos* protein in clone 75/15-2 cells; **E,** expression of TROMA-1 in clone 75/15-2 cells after 3 days induction with 10^{-8} M retinoic acid; **F,** expression of c-*fos* protein in clone 75/15-2 cells after 3 days induction with 10^{-8} M retinoic acid. **Left panels,** phase contrast; **right panels,** fluorescence. Photographic magnification 400x.

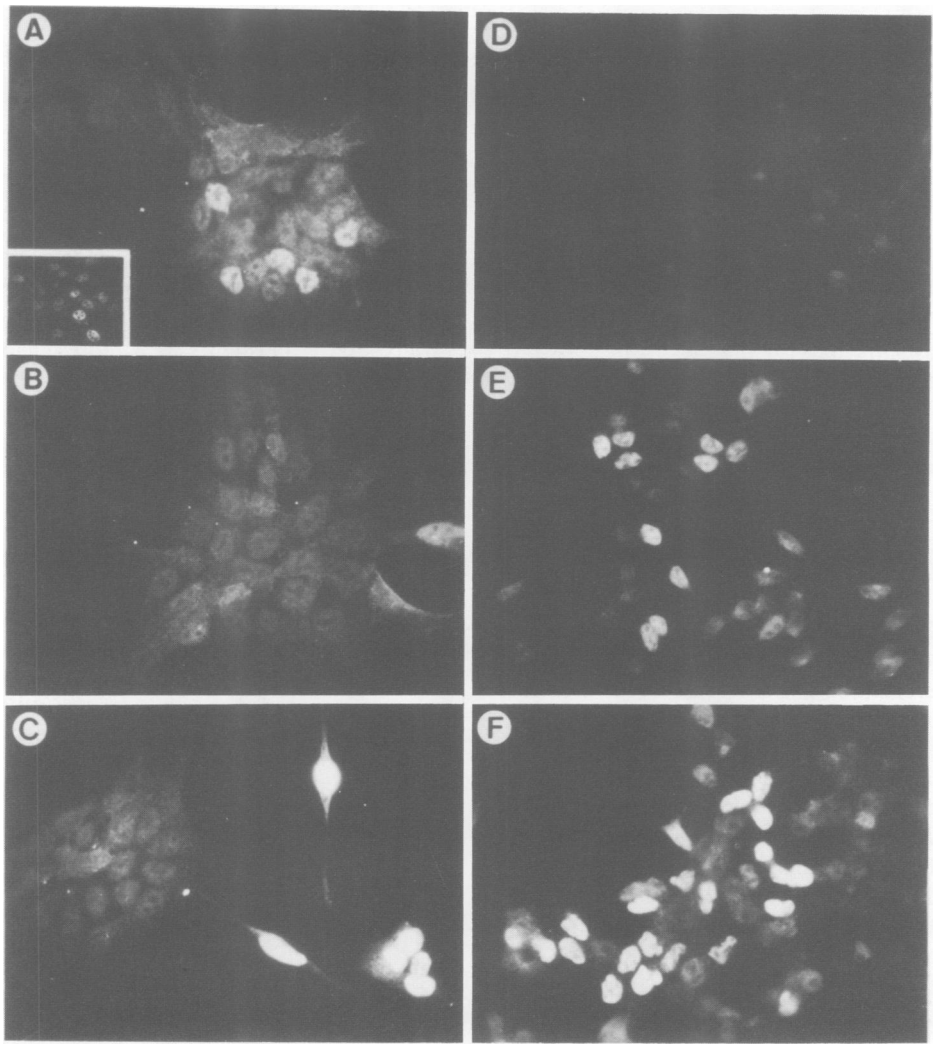

Fig. 7. Immunofluorescence analysis of c-*fos* expression in clones 48/3-3 **(A)**, 75/15-2 **(B,C)** and 76/21-2 **(D,E,F)**. **Panels B** and **C** show different areas of the same slide. **Panels D, E, F** are 76/21-2 cells 0, 2 and 8 h after cadmium induction (5 x 10^{-6} M). In contrast to Figure 6, these pictures were taken under conditions that allow cells with lower levels of expression to be visible. The insert in **panel A** represents fibroblast growth factor (FGF)-stimulated NIH/3T3 cells (1 h of stimulation with 100 μg FGF/ml).

expression (95% of all colonies); (ii) colonies with most of the cells staining strongly positive for TROMA-1 (<1%; Figure 6A); (iii) colonies with only few cells showing strong expression of TROMA-1 (<1%; Figure 6B); and (iv) colonies with most of the cells staining weakly for TROMA-1 (5%; Figure 6C). In normal F9 stem cell cultures, we observed one TROMA-1-positive cell in $>10^4$ cells. These observations led to two main conclusions: (i) a significant percentage of cells transfected with the various c-*fos* gene constructs shows expression of the differentiation-specific marker TROMA-1; and (ii) the fraction of TROMA-1 expressing cells is considerably smaller than the number of morphologically altered cells. Similar results were obtained for the expression of TROMA-3 intermediate filament proteins (data not shown).

When clone 75/15-2 cells were treated with retinoic acid and dibutyryl cAMP for 72 h, cell morphology changed dramatically and >30% of the cells expressed TROMA-1 (Figure 6E), indicating that differentiation to more advanced stages is not blocked in these cells.

Expression of c-fos protein in individual cells

To study the relationship between c-*fos* expression, morphological alteration and differentiation in further detail, expression of c-*fos* protein was analyzed in individual cells by immunofluorescence. Practically all cells of clones 48/3-3 (Figure 7A) and 75/15-2 (Figure 7B) showed significant levels of c-*fos* protein. In these cells, the level of c-*fos* protein was essentially as high as in growth factor-stimulated fibroblasts; where c-*fos* expression is similar to that in virus-transformed cells (Müller *et al.*, 1984b) (see insert in Figure 7A). Some cells of the 75/15-2 population (<1%) showed extremely bright fluorescent staining (Figure 7C). Most of such cells, however, were morphologically indistinguishable from cells of the same clone showing lower expression (Figure 6D; in contrast to Figure 7, this picture was taken through a lens that allowed only high expressor cells to be visible). Similarly, although all cells of clone 48/3-3 showed relatively high c-*fos* protein expression (Figure 7A), a great fraction of clones had stem cell morphology (Figure 2B). With clone 76/21-3, it was not possible to detect any expression of c-*fos* protein by immunofluorescence in the absence of cadmium (Figure 7D) (in agreement with the low mRNA level; Figure 4). Induction with cadmium, however, led to high levels of c-*fos* protein expression within 2 h (Figure 7E) and maximum levels were reached at 6–8 h (Figure 7F). Thereafter, the number of *fos*-positive cells decreased, presumably due to toxic effects exerted by the cadmium. Since practically all cells of clone

76/21-3 were morphologically different from F9 stem cells even in the absence of cadmium, these findings also support the conclusion that expression of relatively low levels of c-*fos* are sufficient to promote a morphological alteration of F9 cells, and that higher levels apparently have no further effect on this phenomenon.

To investigate the question as to whether the extremely high expression of c-*fos* protein in some cells of clone 75/15-2 may be a consequence of cellular differentiation, 75/15-2 cells were also analyzed after induction to differentiation by retinoic acid and dibutyryl cAMP (Figure 6F). These analyses clearly indicated that the fraction of cells showing very high levels of c-*fos* protein remained unchanged, although the majority of the cells showed parameters of cellular differentiation, such as morphological alteration and increased expression of TROMA-1 (in >30% of the cells) (Figure 6E).

Effect of exogenous c-fos genes in other EC cell lines

To investigate whether the promotion of differentiation by c-*fos* might be a F9 cell-specific phenomenon, three of the c-*fos* gene constructs were tested on two other EC cell lines: PC13 (Bernstine *et al.*, 1973) and P19 (McBurney and Rogers, 1981). PC13 cells exhibit similar properties as F9 cells, in that with both cell lines spontaneous differentiation is a very rare event, and both cell lines can be induced by retinoic acid to endodermal differentiation (Rees *et al.*, 1979). P19 cells, on the other hand, are induced by retinoic acid to neural differentiation, whereas dimethyl sulfoxide treatment leads to cardiac muscle differentiation (McBurney *et al.*, 1982). Constructs p19/1 and p48/3 failed to induce any detectable morphological alteration in both P19 and PC13 cells (Table I and data not shown). In contrast, p75/15-transfected P19 cells showed striking morphological alteration associated with the expression of TROMA-1 and TROMA-3 intermediate filament proteins, similar to the morphologically altered c-*fos*-transfected F9 cells (data not shown). PC13 cells, however, showed no effect upon transfection with construct p75/15, although exogenous c-*fos* sequences were expressed at similarly high levels in both p75/15-transfected P19 and PC13 cells (data not shown).

Discussion

Several lines of evidence suggest that the product of the c-*fos* gene plays a role in cellular differentiation processes (Müller *et al.*, 1983,1984a,1985; Müller and Wagner, 1984; Gonda and Metcalf, 1984; Mitchell *et al.*, 1985). The strongest indication for such a role of c-*fos* has been provided by the observation that expression of exogenous mouse or human c-*fos* genes introduced into F9 EC cells results in the appearance of differentiation markers, such as the specific intermediate filament proteins TROMA-1 and TROMA-3 (Müller and Wagner, 1984). To investigate in further detail how c-*fos* expression affects the differentiation state of EC cells we have designed various c-*fos* gene constructs which give rise to different levels of c-*fos* expression and which are inducible upon transfer into F9 EC cells. In the uninduced state, the lowest levels of c-*fos* expression were obtained with constructs p19/1, p48/3 and p76/21 (Table II; Figures 1,3), indicating that neither removal of the 3'-non-translated region of the mouse c-*fos* gene nor the presence of a retroviral LTR down-stream from the c-*fos* coding region significantly influences c-*fos* expression from the metallothionein basal level promoter. Considerably higher levels of constitutive c-*fos* expression were found in clones established after transfection with construct p75/15 (Table II; Figure 3). The relatively high concentrations of c-*fos* mRNA in clones 75/15-2, 75/15-3 and 75/15-4 appears to results at least in part from the high copy numbers of exogenous c-*fos* gene constructs (Table II; Figure 2). This amplification of transfected p75/15 DNA may be due to the presence of the polyoma origin of replication which may lead to replication of the exogenous DNA prior to integration. After induction with cadmium for 6 h, most of the clones analyzed showed a significant increase in c-*fos* mRNA and protein expression (Table II; Figures 3,7). This was particularly noticeable with clones of the 75/15 and 76/21 series where the level of c-*fos* mRNA rose up to 45-fold and >100-fold, respectively (Figure 3, Table II). This observation is intriguing as it represents the first incidence of regulated expression of an exogenous gene introduced into EC cells. It remains to be investigated whether transcription from the inducible metallothionein promoter in F9 EC cells involves an interaction with c-*fos* sequences.

As pointed out in Results and shown in Table II and Figures 3,4,6 and 7, all of the constructs were able to affect the differentiation state of F9 EC cells. Significant numbers of differentiated cells were found even with clones 19/1-1, 48/3-3, 76/21-2 and 76/21-3 (Figure 2; Table II), although c-*fos* expression in these clones was relatively low, i.e. ~1/20 of that in mouse macrophages (Figure 3; Müller *et al.*, 1984a). This indicates that the level required to promote F9 cell differentiation appears to be quite low. Induction of c-*fos* expression by cadmium for up to 10 h followed by 3 days incubation in normal growth medium did not lead to increased numbers of differentiated cells. Two explanations are possible. (i) The period of c-*fos* induction is too short to lead to a biological effect. As longer cadmium treatments are lethal to the cells, this hypothesis cannot be easily tested. (ii) Induction of c-*fos*, even to very high levels (e.g., in clones 75/15-3 or 76/21-3) is insufficient to affect the differentiation state of the entire F9 EC cell population. This hypothesis is lent support by the observation that even in those cell clones showing high levels of constitutively c-*fos* (e.g., clone p75/15-2) only a minor fraction of cells was found to be differentiated (on the basis of morphological alteration and expression of TROMA-1; Figures 3,4,6,7).

Another indication that c-*fos* may act in concert with other event(s) is provided by the observation that cell clones with high c-*fos* expression, but normal stem cell morphology showed morphological alteration and expression of TROMA-1 upon isolation of the clone and replating, even after many passages. It appears that c-*fos* expression in F9 EC cells promotes differentiation only if the cell has undergone some other changes towards differentiation. Evidence for a 'multi-stage' process of c-*fos*-promoted F9 cell differentiation is also provided by the observation that in clones isolated after transfection with c-*fos* gene constructs the fraction of morphologically altered cells was considerably greater than that of TROMA-1-positive cells (Figures 4,6). A 'multi-stage' hypothesis would be in agreement with the result that the effect of c-*fos* is more efficient in F9 cells than in P19 cells and is undetectable in PC13 cells (Table I). P19 cells may require a higher expression of c-*fos* to show a biological effect, since only construct p75/15 was able to induce morphological alteration and showed expression of TROMA-1. It has previously been reported that F9 cells have a less pronounced stem cell character than P19 and PC13 cells in that F9 cell cultures show higher levels of epidermal growth factor receptor and transferrin expression than both other EC cell lines (Adamson and Hogan, 1984). The lowest expression of these differentiation markers was observed in PC13 EC cell cultures (Adamson and Hogan, 1984).

In conclusion, our results confirm earlier observations that c-*fos* expression can promote the differentiation of certain EC cell lines, but it appears that expression of the c-*fos* gene product alone is insufficient to induce the process of cellular differentiation. Other events seem to be required to complement the differentiation-promoting properties of c-*fos*.

Materials and methods

DNA construction

To facilitate subsequent steps, the *Eco*RI site in the first intron of the mouse c-*fos* gene was destroyed by filling-in using the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer, Mannheim) followed by blunt-end ligation. As determinated by sequence analysis (Maxam and Gilbert), both ends of the restriction site were filled in in plasmid p3/4. The *Nae*I site at position +41 in the c-*fos* gene was changed into a *Bam*HI site by subcloning of the 5′ *Nae*I-*Xba*I c-*fos* gene fragment of p3/4 into the *Hind*II-*Xba*I sites of pUR250 (Rüther, 1982). This *Bam*HI-*Xba*I fragment, together with the 3′ *Xba*I-*Bam*HI c-*fos* gene fragment, was cloned into the *Bam*HI site of pHSl, a plasmid carrying the human metalliothionein promoter region (−770 to +75) (Karin *et al.*, 1984). From this new plasmid, designated p15/2, a *Hind*III-*Eco*RI fragment containing the whole metalliothionein promoter as well as the c-*fos* coding region including the translation stop codon, poly(A) addition site and ~800 bp of flanking sequence was cloned into the *Bam*HI and *Eco*RI site of the pSV2-*neo* vector (Southern and Berg, 1982). The *Hind*III and the *Bam*HI site were made compatible by a DNA polymerase I fill-in reaction. The generated plasmid was designated p19/1 (Figure 1). Plasmid p48/3 was created by deletion of a *Sal*I-*Bcl*I fragment of 478 bp in the 3′-non-coding region of c-*fos*. Both restriction sites were made compatible for blunt-end ligation by DNA polymerase I fill-in reaction. Plasmid p75/15 was constructed by cloning of the *Eco*RI-*Bam*I fragment of pPyLTl (Rassoulzadegan *et al.*, 1983) into the *Eco*RI-*Bam*HI sites of p19/1. Plasmid p76/21 was constructed in two steps. First, the 3′ LTR of pFBJ-2 (Curran *et al.*, 1982; Van Beveren *et al.*, 1984) was subcloned as a *Bam*HI-*Sal*I fragment into pUC19 (Norrander *et al.*, 1984). This fragment was exchanged with the *Eco*RI-*Sal*I fragment of p19/1 (Figure 1).

Cell lines and transfection

F9 cells, PC13 cells (Bernstine *et al.*, 1973) and P19 cells (McBurney and Rogers, 1981) were obtained from E. Adamson, La Jolla, California. For transfection, 5 − 10 μg plasmid DNA per 10^6 cells were co-precipitated without carrier DNA as described (Graham and van der Eb, 1973; Müller and Wagner, 1984). Following selection in G418, individual colonies were picked and expanded. In several cases (e.g., clone 19/1-2 in Figure 2) the clonal origin of the cells was confirmed by Southern analysis of transfected sequences.

Isolation of genomic DNA and cytoplasmatic RNA

About 10^8 cells were collected by trypsinization, washed once with phosphate-buffered saline (PBS) and then lysed with 0.5% Nonidet P-40 (NP-40) in 10 mM Tris-HCl pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$ and 10 mM EDTA for 5 min at 0°C. After centrifugation for 5 min at 3000 r.p.m. the supernatant was extracted once with hot phenol/0.5% SDS at 65°C and once with phenol/chloroform. The RNA was precipitated at −20°C overnight after addition of 1/10 volume of 2 M NaAc, pH 5.5 and two volumes of ethanol. The precipitate was collected by centrifugation and dissolved in 100 μl H_2O. The pellet of the NP-40-lysed cells containing the nuclei was incubated overnight in 1% SDS and 25 μg proteinase K/ml followed by a phenol/chloroform extraction. High mol. wt. DNA was recovered by ethanol precipitation. Southern blots were performed as described (Southern, 1975).

RNAs were separated in formaldehyde-containing agarose gels, transferred to nitrocellulose paper and hybridized to nick-translated probes as described (Lehrach *et al.*, 1977; Müller *et al.*, 1984b).

Protein analyses

For immunoprecipitation, cells on 25 cm^2 dishes were labeled for 5 h with 7000 μCi of [^{35}S]methionine (Amersham) in 1 ml of methionine-free medium supplemented with 10% dialysed fetal calf serum. Cells were lysed and immunoprecipitation was carried out as described (Müller *et al.*, 1984). Immunofluorescence analyses were performed with either *p*-formaldehyde-fixed cells (*fos*) or with ethanol-acetic fixed cells (TROMA-1) as previously reported (Curran *et al.*, 1984; Müller and Wagner, 1984).

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