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Wounding a fibroblast monolayer results in the rapid induction of the c-*fos* proto-oncogene

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The c-*fos* gene has previously been shown to be transiently induced within minutes after the stimulation of mouse fibroblasts with growth factors. Induction of c-*fos* was observed specifically with competence factors (e.g., platelet-derived growth factor), not with progression factors (e.g., platelet-poor plasma), suggesting a role for c-*fos* in conferring competence on fibroblasts. To test this hypothesis we have analyzed c-*fos* expression in NIH 3T3 cells that were made competent in a different way, namely by wounding a confluent monolayer of cells. Using antibodies raised against either a synthetic *fos* peptide or a β-galactosidase–*fos* fusion protein, we show in this study that in the majority of cells lining the wound c-*fos* protein is rapidly and transiently induced to high levels. No induction is observed in cells at a distance from the wound greater than ~5 cell layers. Induction is equally efficient in both serum-containing and serum-free medium, and is similar in cells that were deprived of fetal calf serum for 40 h prior to making the wound. Our observations support the hypothesis that c-*fos* may be involved in inducing the 'competent state' in fibroblasts and suggests an early role for c-*fos* in wound healing and tissue regeneration.

Key words: c-*fos*/fibroblast monolayer/wounding/competence

Introduction

The operational definitions of 'competence' and 'progression' have been introduced to distinguish different phases in the growth response of 3T3 mouse fibroblasts to certain growth factors (Stiles *et al.*, 1979). Quiescent (serum-deprived) mouse fibroblasts exposed for a short time to platelet-derived growth factor (PDGF) become 'competent' for growth (Stiles *et al.*, 1979). Exposure to platelet-poor plasma (PPP), which has no detectable effect on the growth behaviour of untreated cells, allows competent cells to progress through G1 and to enter the S-phase. A role for the c-*fos* and c-*myc* proto-oncogenes in inducing the 'competent state' in fibroblasts is suggested by the observation that both genes are rapidly induced by all competence factors tested [e.g. fetal calf serum (FCS), PDGF or fibroblast growth factor] (Kelly *et al.*, 1983; Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Müller *et al.*, 1984; Cochran *et al.*, 1984) but not by the progression factors contained in PPP (Bravo *et al.*, 1985a). Epidermal growth factor (EGF) is a polypeptide that cannot be classified in the scheme of competence and progression, in that 3T3 cells treated for a short time with EGF do not respond to PPP. In contrast, the continuous exposure of NIH 3T3 cells to EGF takes almost half of the cell population to the S-phase (Müller *et al.*, 1984). It has been shown that EGF induces c-*fos* in NIH 3T3 cells with the same kinetics as PDGF, but to a much lesser extent (Müller *et al.*, 1984). This finding is in agreement with the conclusion that a high induction of c-*fos* is specific for competence factors. It can, however, not be ruled out that the induction of c-*fos* may also play a role in growth-controlling mechanisms other than the induction of competence.

Recent studies have shown that *myc*-expressing fibroblasts are able to enter S-phase in PPP (Armelin *et al.*, 1984), and microinjection of *myc* protein has been reported to render quiescent fibroblasts competent for growth (Kazmareck *et al.*, 1985). In contrast, c-*fos*- or v-*fos*-transformed fibroblasts are dependent on the presence of PDGF for growth (R.Bravo and R.Müller, unpublished observation). This observation suggests that if c-*myc* is involved in inducing the 'competent state', then it must act in concert with other mechanisms. This, however, applies also to the role of c-*myc*, since growth factor dependence is not completely abbrogated in *myc*-transformed cells (Armelin *et al.*, 1984). To test this hypothesis for the c-*fos* gene we have used another system previously described for the induction of competence in mouse fibroblasts. It has been shown that quiescent BALB/c 3T3 fibroblasts enter the cell cycle and reach S-phase when a confluent monolayer is wounded in the presence of PPP (Stiles *et al.*, 1979) indicating that wounding is equivalent to treatment with a competence factor. We have therefore analyzed the effect of wounding a confluent monolayer of NIH 3T3 cells on the expression of the c-*fos* proto-oncogene.

Results and Discussion

Expression of β-gal–fos protein and generation of fos-specific antibodies

Two different types of antisera were used in the present study. An antiserum against a peptide present in both viral and cellular *fos* gene products (KEKEKLEFIL) has previously been described (Müller *et al.*, 1986). Since only limited quantitites of this antiserum obtained from a single rabbit are available and the quality of anti-peptide sera obtained from several other rabbits varied enormously (in particular with respect to their ability to recognize the native c-*fos* protein) we decided to raise antibodies against a β-galactosidase–(β-gal-)*fos* fusion protein covering the amino acid sequence used for generation of the anti-peptide serum. For this purpose a *Pst*I–*Ava*II fragment of FBJ-MSV (nucleotides 1794–2221 covering parts of exons 3 and 4 of c-*fos*; Van Beveren *et al.*, 1983; see Figure 1a) was cloned into the expression vector pEX2 (Stanley and Luzio, 1984). Figure 1b (lane 3) shows the synthesis of a protein of ~135 kd in bacteria transformed with this construct. The mol. wt of this protein was clearly increased compared with normal β-gal protein (116.5 kd; Figure 1a, lane 2) and corresponded to the expected calculated value. The 135-kd protein reacted with the *fos* peptide antibodies (Figure 1c) indicating that it represents a β-gal–*fos* fusion protein. This protein was used for the production of antibodies in three rabbits. After one boost injection, all antisera had high titers of antibodies specifically precipitating the c-*fos* gene product from clone 5-3a cells (expressing exogenous c-*fos* protein; see Figure 2 legend for details) and from

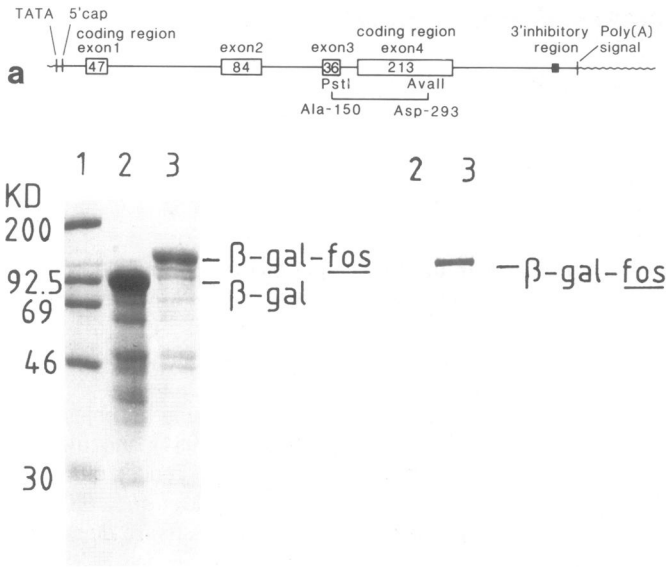

Fig. 1. Expression of β-gal−*fos* protein. (**a**) Structure of the mouse c-*fos* gene. The position of the *Pst*I−*Ava*II fragment (from FBJ-MSV to exclude the intron) used for construction of the β-gal−*fos* expression vector is shown. Numbers indicate amino acids. (**b**) Coomassie-stained polyacrylamide gel. **Lane 1**, marker proteins; **lane 2**, lysate from bacteria expressing normal β-gal; **lane 3**, lysate from bacteria expressing β-gal−*fos* fusion protein. (**c**) 'Western blot' analysis of **lanes 2** and **3** of the gel shown in **a**, using *fos*-peptide antibodies (Müller *et al.*, 1986).

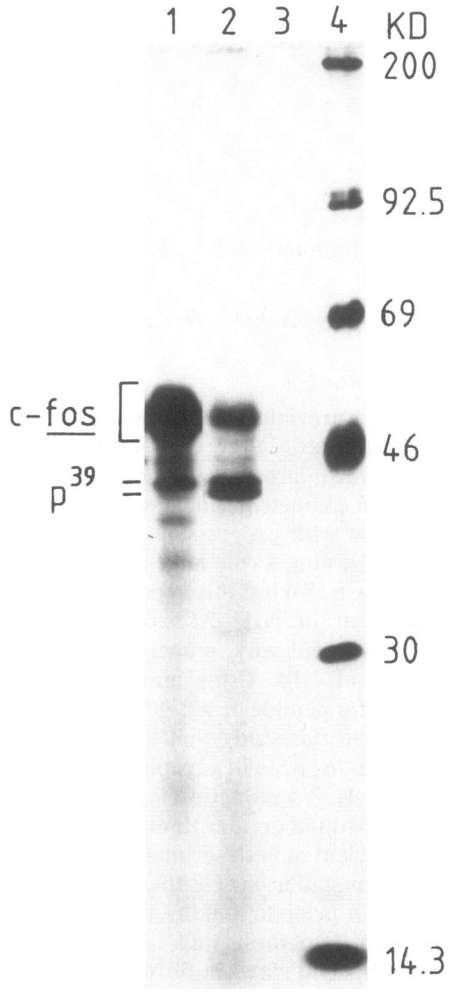

Fig. 2. Precipitation of c-*fos* protein by antibodies raised against β-gal−*fos* fusion protein. **Lane 1**, immunoprecipitation of c-*fos* protein from clone 5-3a cells which express the c-*fos* gene product from a transfected plasmid (p76/2); carrying a metallothionein promoter-driven mouse c-*fos* gene; (Müller *et al.*, 1986). The antiserum was analyzed after immunization and one boost injection with the β-gal−*fos* fusion protein (antiserum α-*fos*-454). Cells were labelled for 15 min with 0.3 mCi [^{35}S]- methionine/ml in medium lacking unlabeled methionine. **Lane 2**, immunoprecipitation of c-*fos* from serum-stimulated NIH 3T3 cells using the same serum as in **lane 1**. Cells were serum-saved for 20 h and stimulated with FCS for 1 h. During the last 15 min of stimulation the cells were labeled with 0.7 mCi [^{35}S]methionine/ml in medium lacking unlabeled methionine. **Lane 3**, the same lysate is used in **lane 2** was precipitated with a rabbit non-immune serum. **Lane 4**, marker proteins. No ^{35}S-labeled protein was precipitated with the same antibodies under the same experimental conditions from quiescent NIH 3T3 cells (not shown).

serum-stimulated NIH 3T3 fibroblasts (Figure 2). As previously described, c-*fos* protein was modified and complexed with the cellular protein p39 (Müller *et al.*, 1984; Curran *et al.*, 1985). In addition, the antibodies recognized c-*fos* protein in immunofluorescence experiments (data not shown; see also Figure 3 and 4). These observations show that the antisera produced by immunization with the β-gal−*fos* fusion protein represents reproducible, specific reagents for the detection of c-*fos* protein.

Induction of c-fos in wounded monolayers

The immunofluorescence analysis in Figure 3 shows that within 1 h after scratching a line into a confluent monolayer of NIH 3T3 cells c-*fos* protein was detected at high levels specifically in cells lining the wound. Expression of c-*fos* protein gradually decreased with distance from the wound, and was undetectable in cells at a distance greater than ~5 cell layers, suggesting that intercellular communication may play a role in signal transduction. The level of c-*fos* expression in cells closest to the wound was very similar to that observed after growth factor stimulation of quiescent NIH 3T3 cells (Müller *et al.*, 1984; R.Müller, unpublished observation). Expression of c-*fos* protein remained high for another hour, then gradually decreased and was barely detectable after 5 h. At 24 h c-*fos* protein was undetectable in all cells (Figure 3). This time course closely resembles the kinetics of c-*fos* induction in growth factor-stimulated NIH 3T3 fibroblasts (Müller *et al.*, 1984).

Induction of c-fos in the absence of serum growth factors

To test the possibility that induction of c-*fos* may be a consequence of an increased cell surface area exposed to serum growth factors in the area of the wound, the wound assay was carried out in serum-free medium (Dulbecco's modified Eagle's medium, DMEM). For this purpose, confluent NIH 3T3 monolayers were rinsed with DMEM and then kept in serum-free medium for varous times (0−24 h) prior to wounding the monolayer. Figure 4 shows that c-*fos* was induced in all cases to similar levels and in a similar fraction of cells as in the presence of serum (compare with Figure 3). The experiment was also carried out under more stringent conditions: monolayers were pre-incubated twice for 10 min in serum-free medium, then kept for another 40 h in medium without serum, rinsed again with serum-free medium and wounded by scratching a line. Again, c-*fos* protein was induced in cells lining the wound with similar efficiency as in the presence of FCS (Figures 3 and 4). It has previously been demonstrated that the level of c-*fos* induction by growth factors is a function of the growth factor concentration in BALB/c 3T3 cells (Kruijer *et al.*, 1984), in A431 cells (Bravo *et al.*, 1985b)

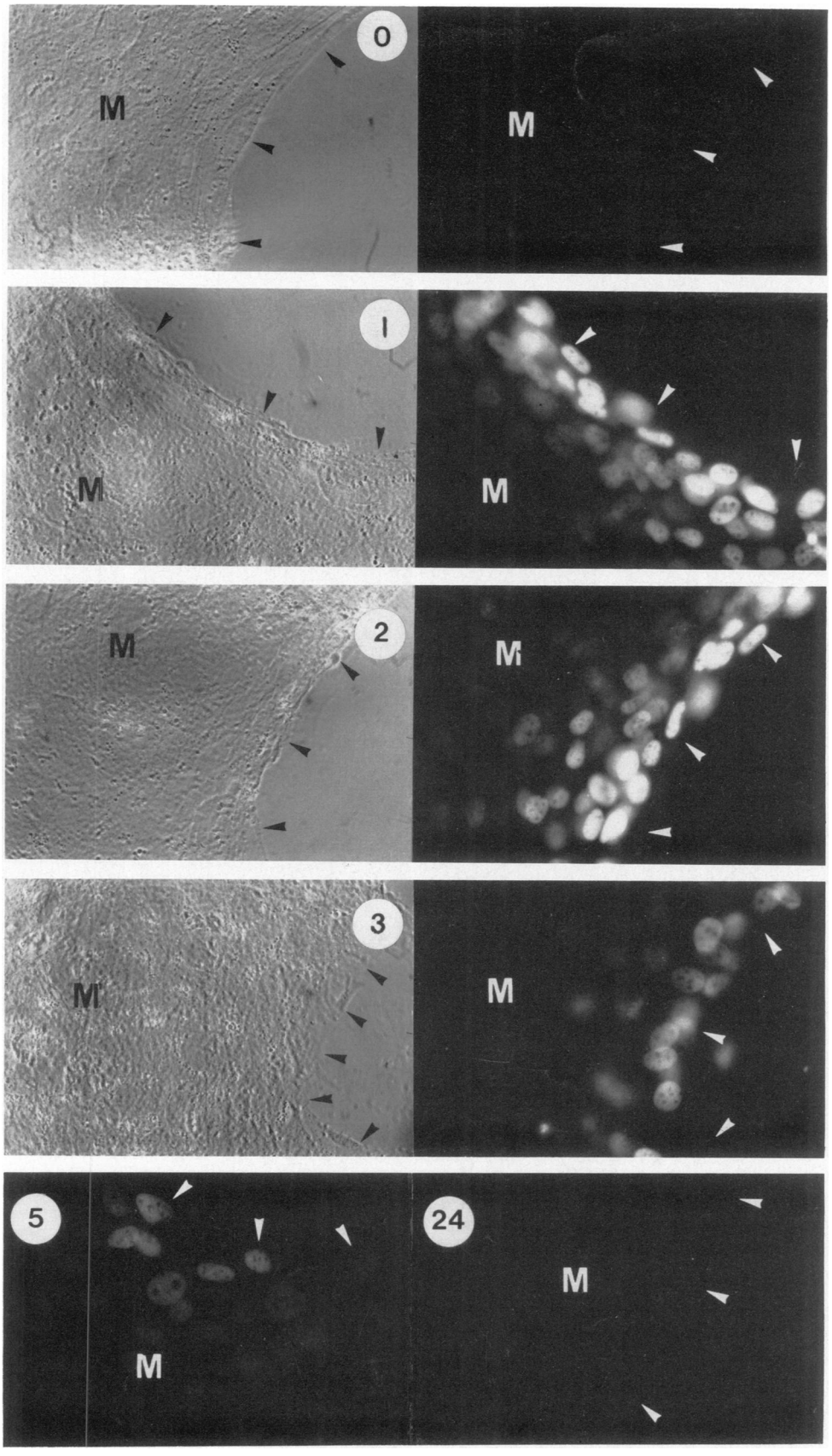

Fig. 3. Kinetics of c-*fos* protein induction following wounding of a confluent NIH 3T3 monolayer in the presence of FCS. NIH 3T3 cells were grown to confluence in DMEM supplemented with 10% FCS. The last medium change was performed just prior to the experiment. Monolayers were wounded by scratching lines with a plastic pipette tip, fixed in paraformaldehyde 0, 1, 2, 3, 5 and 24 h later and analyzed by immunofluorescence (see Materials and methods). **Left panels** (at 0, 1, 2, and 3 h time points), phase contrast; **right panels**, fluorescence. Arrows point to the margin of the wounded monolayer; M, monolayer. Identical results were obtained with antisera directed against either β-gal–*fos* fusion protein or synthetic *fos* peptide (not shown).

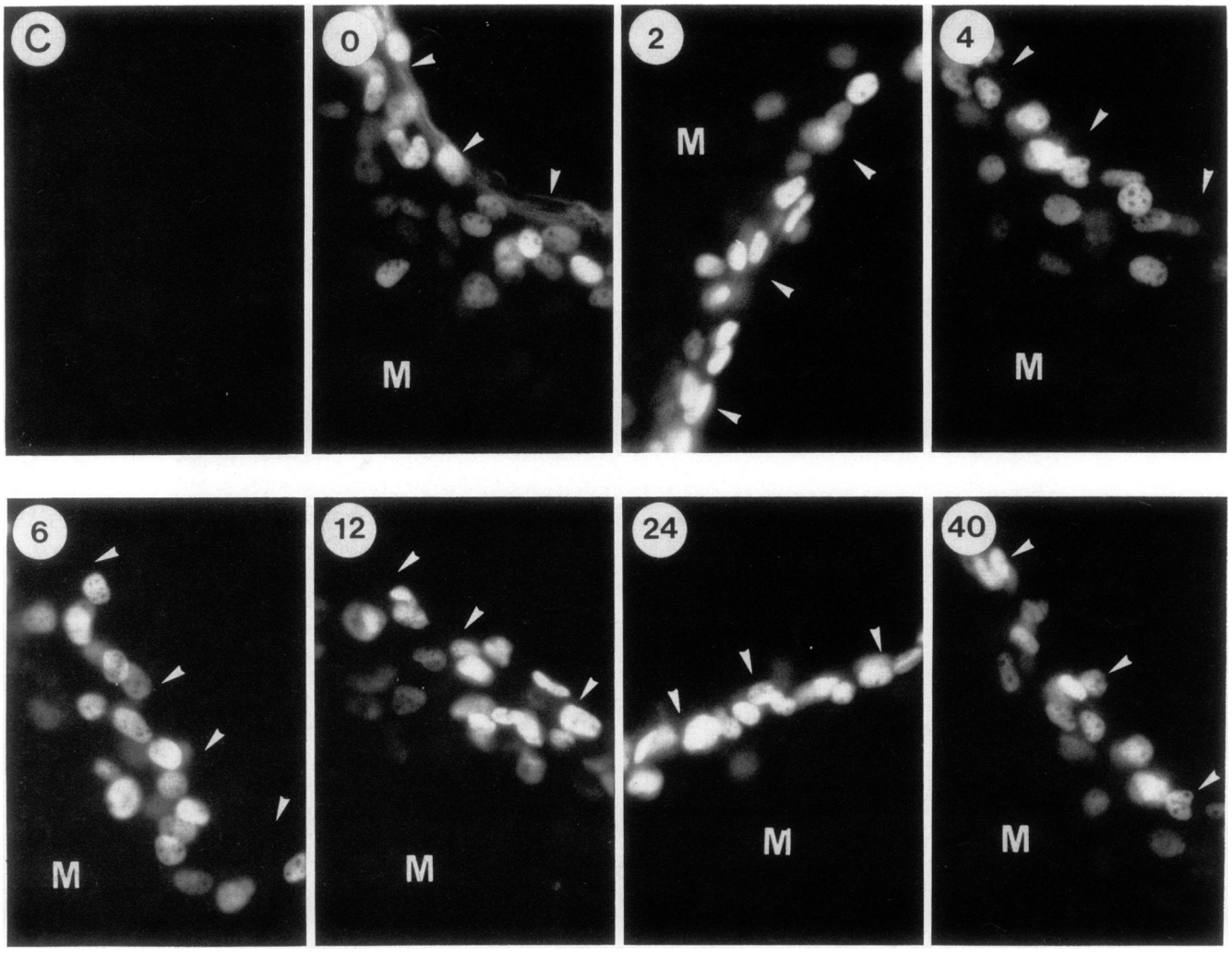

Fig. 4. Induction of c-*fos* protein after wounding a confluent monolayer of FCS-deprived NIH 3T3 cells. The experiment was performed as described in the legend to Figure 1, except that the last medium change was done 1 day prior to the experiment, and the cells were deprived of FCS for 0, 2, 4, 6, 12 or 24 h, as indicated, prior to making the wound. At the time of switching to serum-free medium, cultures were rinsed with DMEM without serum and, in the case of the 40 h time point, pre-incubated with DMEM twice for 10 min at 37°C to remove residual serum growth factors as thoroughly as possible. After the 40 h incubation period, the medium was changed again prior to making the wound. Monolayers were fixed 1 h after wounding. C, normal NIH 3T3 monolayer.

and in NIH 3T3 cells (R.Müller and R.Bravo, unpublished observation). If c-*fos* induction in the wound assay was a consequence of residual serum growth factors, then one should expect to see clearly different levels of expression depending on whether the monolayer was wounded in the presence of FCS, in serum-free medium or after incubation in serum-free medium for 40 h. This, however, is clearly not the case, suggesting that in the wound assay c-*fos* is not induced by serum growth factors.

Synthesis of modified c-fos protein

To demonstrate the induction of c-*fos* protein not only by immunofluorescence staining, the assay was performed in such a way as to allow the induction of c-*fos* in the majority of cells in the culture. For this purpose, confluent monolayers were scraped from the culture dish, the cells were suspended by pipetting up and down and finally plated at a lower density without changing the medium. At various times after replating, the cells were harvested and analyzed by Western blotting (Figure 5). As expected, c-*fos* protein was clearly detectable 1 h after plating the cells, and expression was very low at 4 h. It has previously been described that immunoprecipitated c-*fos* protein in growth factor-stimulated NIH 3T3 cells is post-translationally modified (Kruijer *et al.*, 1984; Müller *et al.*, 1984). Likewise, the protein blot analysis displayed in Figure 5 shows highly modified c-*fos* protein in the range of 55–62 kd.

Conclusions

Our observations show that wounding of a confluent NIH 3T3 monolayer results in the rapid, transient induction of c-*fos* protein expression, which apparently occurs independently of serum growth factors. The mechanism responsible for c-*fos* induction may involve alterations in the plasma membrane, an increased interaction with fibroblast-secreted molecules, a disruption of intercellular communication or other events. However, investigating the precise nature of the induction mechanism will be the subject of further study.

Taken together with other observations, several lines of evidence suggest a role for c-*fos* in the induction of competence. Firstly, c-*fos* is specifically induced by competence factors (Bravo *et al.*, 1985a). Secondly, mouse fibroblasts made competent by wounding a monolayer show c-*fos* protein induction specifically

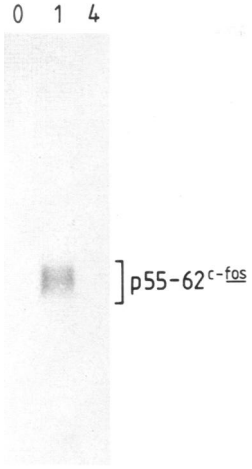

Fig. 5. Detection of c-*fos* protein in NIH 3T3 cells released from density arrest. A confluent NIH 3T3 monolayer grown in DMEM plus 10% FCS was scraped from the dish using a rubber policeman, cells were suspended by pipetting up and down and seeded in another dish at 60% of the original density. All stages were carried out without medium change, i.e. scraping, suspending and plating were performed in the original medium. Cells were harvested 0, 1 and 4 h after re-seeding and lysed in SDS-sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions followed by 'Western blot' analysis as previously described using *fos* peptide antiserum (Müller *et al.*, 1986).

in cells lining the wound. Thirdly, the induction of competence is possible at different stages of the cell cycle (Scher *et al.*, 1979). Accordingly, we have recently shown that c-*fos* is inducible throughout the cell cycle (except for mitosis) (Bravo *et al.*, 1986). It should, however, be stressed that all these pieces of evidence do not prove a role for c-*fos* in the induction of competence. However, it is extremely difficult at present to obtain unequivocal data for such a role of c-*fos*, in particular since *fos*-expressing fibroblasts are completely dependent on 'competence' factors (e.g. PDGF) for growth. Since also *myc*-expressing cells are not fully competent (Armelin *et al.*, 1984) *fos* and *myc* gene products may cooperate in the induction of competence, perhaps with other members of the '*fos* family', such as r-*fos* (Cochran *et al.*, 1984) or with other gene products induced by growth factors (Cochran *et al.*, 1983; Lan and Nathans, 1985). It is thus possible that the induction not only of c-*myc*, but also of c-*fos*, is an event that normally occurs during the recruitment of quiescent cells *in vivo*, such as in wound healing and tissue repair. However, the analysis of the precise function that nuclear proto-oncogene products play in the control of cell proliferation in general, and more specifically in the induction of 'competence', has to await the isolation and characterization of the other genes induced by growth factors or other growth stimuli.

Materials and methods

Cell culture

NIH 3T3 cells (clone 7; obtained from D.Lowy, NIH) were grown in DMEM supplemented with 10% FCS. Serum stimulation of quiescent cells was carried out as previously described.

Expression of β-gal–fos fusion protein in Escherichia coli

A *TaqI–AvaII* fragment of FBJ-MSV (nucleotides 1340–2221) was subcloned into the *SmaI* site of pUC18 (Norrander *et al.*, 1983) by blunt end ligation (construct kindly provided by T.Jenuwein), excised with *PstI* and a fragment containing 427 bp of FBJ-MSV (nucleotides 1794–2221) was inserted into pEX2 (Stanely and Luzio, 1984) and used for transformation of *E. coli* K-12ΔHIΔtrp. Cells were grown and heat-induced exactly as described (Stanley and Luzio, 1984).

Immunization of rabbits

Bacterial lysate was subjected to polyacrylamide gel electrophoresis and the band containing the β-gal–*fos* protein was excised. The gel pieces were homogenized in phosphate-buffered saline (PBS), mixed with 1 vol of complete Freund's adjuvant and injected into the rabbits (200 μg of protein/animal). Ten weeks later the rabbits were boostered by injection of 200 μg of β-gal–*fos* fusion protein (isolated by electroelution from polyacrylamide gel pieces; Knowles and Bologna, 1983), emulsified in incomplete Freund's adjuvant. Blood was taken 10 days later, and boost injections were repeated at 4-week intervals.

Immunochemical and immunocytological detection of c-fos protein

Briefly, p-formaldehyde-fixed and Triton X-100-permeabilized cells were stained with either an anti-*fos* peptide serum (raised against a conjugate of KEKEKLEFIL and keyhole limpet hemocyanin; Müller *et al.*, 1986) at a dilution of 1:200 or with the antiserum described in this study (at a dilution of 1:100), followed by rhodamine-conjugated sheep anti-rabbit IgG (Dakopatts; used at a dilution of 1:20). Otherwise, immunofluorescence experiments were carried out as previously described (Müller *et al.*, 1984, 1986). Western blotting and immunoprecipitation were performed essentially as reported elsewhere (Müller *et al.*, 1984, 1986).

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