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Expression of c-*fos* in NIH3T3 cells is very low but inducible throughout the cell cycle

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It has previously been shown that the c-*fos* proto-oncogene is rapidly and transiently induced following growth factor stimulation of quiescent NIH3T3 mouse fibroblasts. To investigate a possible role of c-*fos* in growth control mechanisms we have studied its expression and inducibility during the NIH3T3 cell cycle. Two major conclusions can be drawn from this analysis. First, expression of c-*fos* is not cell cycle-regulated, and is barely detectable in all phases of the cycle. Second, cells at different stages of the cell cycle (except for mitosis) are as sensitive to c-*fos* induction by growth factors as quiescent cells. These observations suggest that induction of the c-*fos* gene does not play a role during the continuous cycling of NIH3T3 cells, but they are fully compatible with the hypothesis that a function of c-*fos* may be associated with the induction of competence in fibroblasts. Through such a function c-*fos* may contribute to moving cells out of the quiescent state.

Key words: proto-oncogenes/growth factors/cell synchronization/competence/cell proliferation

Introduction

Among the earliest known events following stimulation of quiescent fibroblasts by peptide growth factors is the transient induction of c-*fos* and c-*myc* proto-oncogenes (Campisi *et al.*, 1984; Cochran *et al.*, 1984; Greenberg and Ziff, 1984; Kelly *et al.*, 1983; Kruijer *et al.*, 1984; Müller *et al.*, 1984). This observation has led to the hypothesis that the c-*fos* and c-*myc* gene products play important roles in the control of cell proliferation. Such functions of c-*fos* and c-*myc* could be associated with signal transduction occurring during the normal cell cycle (for instance in the early G1 phase), or, alternatively, being found only in special conditions, such as the transition from the quiescent (G0) state to the G1 phase. Recently, it has been demonstrated that the levels of c-*myc* RNA and protein are invariable throughout the cell cycle in chicken fibroblasts, as well as in human lymphocytes, leukemia cells and HeLa cells (Hann *et al.*, 1985; Rabbitts *et al.*, 1985; Thompson *et al.*, 1985), but data on the expression of c-*fos* during the cell cycle have not been reported to date. Thus, it remains unclear whether induction of c-*fos* may play a role in the normal cycling of fibroblasts. The first goal of this study was, therefore, to study the expression of the c-*fos* proto-oncogene during the cell cycle in NIH3T3 cells.

Growth factors can act as either 'competence factors' or 'progression factors' (Stiles *et al.*, 1979). If fibroblasts are exposed to a competence factor (e.g. platelet-derived growth factor, PDGF) for a short time, the cells become 'competent' for growth in that upon subsequent treatment with a progression factor (e.g. platelet-poor plasma, PPP) they proceed through G1 and reach the S-phase. Treatment of non-competent cells with PPP alone has no detectable effect on the growth state of the cells. The c-*fos* gene is induced only by competence factors [e.g. fetal calf serum (FCS), PDGF, fibroblast growth factor (FGF)], but not by the progression factors in PPP (Bravo *et al.*, 1985). These observations suggest that the induction of c-*fos* may play a role during the induction of competence in fibroblasts, although a high c-*fos* expression is apparently not required for maintenance of the component state (Bravo *et al.*, 1985). If this hypothesis is correct, then c-*fos* should be inducible at various stages of the cell cycle, since it has been shown that fibroblasts can be made competent not only in the quiescent state but also in the S-phase (Scher *et al.*, 1979). The second aim of this investigation was, therefore, to study the inducibility of the c-*fos* gene at various stages of the NIH3T3 cell cycle.

Results

c-fos expression during the cell cycle

To produce synchronous G1 populations mitotic cells, obtained as described in Materials and methods, were released from the nocodazole block by plating in normal Dulbecco's modified Eagle's medium (DMEM) plus 10% FCS. As shown by thymidine labelling followed by autoradiography, the cell population obtained in this way synchronously reached the S-phase after ~5 h (Figure 1a), indicating that the cells had entered the G1-phase shortly after nocodazole removal. RNA was isolated at different times after release from mitosis and analyzed for c-*fos* expression. Figure 1b shows that the level of c-*fos* mRNA in both mitotic and G1 cells was considerably lower than that found in growth factor-stimulated quiescent cells. The small increase in the level of c-*fos* expression shortly after release from the nocodazole block (Figure 1b) is probably a consequence of plating the cells in fresh medium, since a similar increase in c-*fos* expression was observed in exponentially growing cells and in S-phase cells after changing the medium (R.Bravo and R.Müller, unpublished results). The experiment was therefore repeated using, for replating, the mitotic cell's (after the nocodazole block) 'conditioned' medium (i.e. the medium in which the cells were grown prior to switching to nocodazole-containing medium). Under these conditions, expression of c-*fos* was barely detectable at all times after replating, and no increase shortly after release from the nocodazole block was observed (Figure 1c). This indicates that c-*fos* expression is absent or remains very low as cells proceed through G1 and reach the S-phase (Figure 1b, late time points).

To study the expression of c-*fos* during the S-phase, cells were blocked in the G1/S boundary with hydroxyurea as described in Materials and methods. Hydroxyurea removal allowed the cells to move synchronously into the S-phase (Figure 2a) and to complete cell division, even in the absence of serum (unpublished

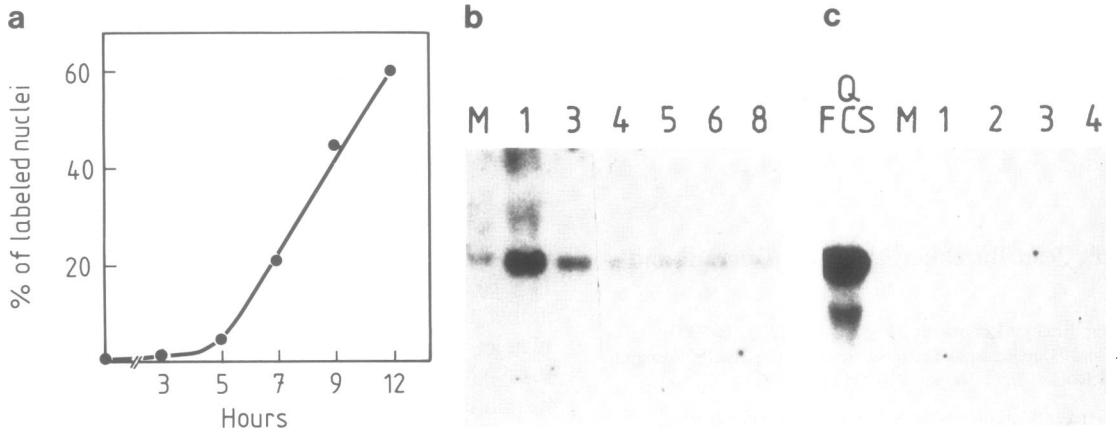

Fig. 1. c-*fos* expression during G1. (**a**) Mitotic cells plated on coverslips were labelled for 30 min with [^{3}H]thymidine at the indicated times and processed for autoradiography. About 500 nuclei were counted in each case. (**b,c**) RNA blot analysis of c-*fos* RNA from cells in mitosis (M) and during G1. **b**, mitotic cells were plated in fresh medium (see Materials and methods); **c**, mitotic cells were plated in 'conditioned' medium. Numbers indicate hours after plating of mitotic cells. Q/FCS, quiescent cells stimulated with 10% of FCS for 1 h.

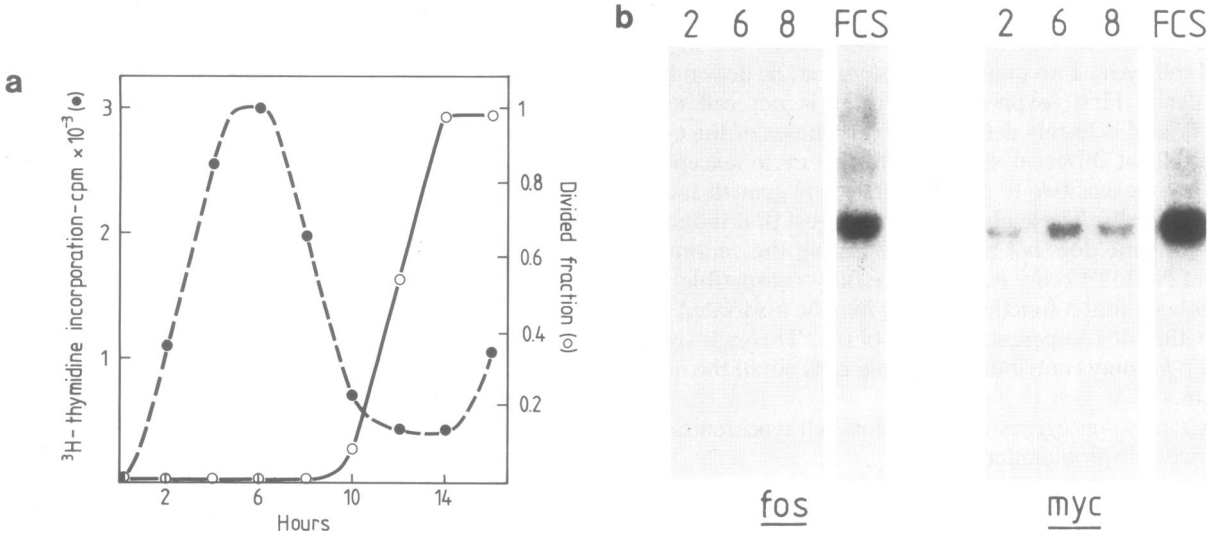

Fig. 2. Expression of c-*fos* during S-phase. Quiescent cells were stimulated with 10% FCS in the presence of 2 mM hydroxyurea for 18 h. The hydroxyurea was removed and cells were incubated at 37°C in fresh medium. (**a**) Cells were labelled for 2 h with [^{3}H]thymidine at each indicated time and incorporated radioactivity was determined as described (Macdonald-Bravo and Bravo, 1985). Divided fraction represents $N/N0-1$ so that a true doubling of the cell population would appear as an increase from 0 to 1.0 on the ordinate axis. N = number of cells at any given time in the experiment; $N0$ = initial cell number. (**b**) Cells were analyzed for c-*fos* and c-*myc* expression by RNA blot analysis. FCS, quiescent cells induced with FCS for 1 h.

observations). The fraction of cells entering the S-phase was >90%, as determined by autoradiography (data not shown). analysis of RNA prepared at different times after hydroxyurea removal showed that c-*fos* expression was extremely low, if detectable at all, throughout the S-phase (Figure 2b). For comparison, the expression of c-*myc* RNA at this stage of the cell cycle is also shown in Figure 2b. The levels of c-*myc* mRNA did not show significant fluctuations during the S-phase and was several fold lower than the maximal expression observed in quiescent stimulated cells. Likewise, c-*fos* mRNA expression in G2 cells was practically undetectable under the assay conditions used (data not shown). These observations demonstrate that the extremely low levels of expression of c-*fos* RNA do not significantly change during mitotis, G1, S-phase and G2 in NIH3T3 cells.

Induction of c-fos and c-myc in serum-deprived asynchronous cultures

As the high levels of c-*fos* expression in stimulated quiescent cells could not be explained as an event that occurs normally during the G1 phase, we attempted to obtain further evidence for the hypothesis that the induction of c-*fos* by growth factors may play a role in conferring competence on fibroblasts. We therefore investigated whether c-*fos* is inducible at various stages of the cell cycle. Exponentially growing cells were serum-deprived for 2, 4, 6, 8, 10, 12 or 24 h, and at each time point 10% FCS was added for 1 h. Autoradiography analysis showed that the thymidine labelling index after 6 h of serum deprivation had only slightly declined from 52% to 45% (Figure 3a). A significant decrease in the thymidine labelling index was apparent after 8 h (27%) of serum deprivation. As shown in Figure 3b, maximal induction of the proto-oncogenes was observed after 6 h of serum deprivation, at a time when the thymidine labelling index had not yet significantly changed. Similar results were obtained with c-*myc* (Figure 3b). No significant differences in the levels of c-*fos* and c-*myc* induction were found when cells were serum-deprived for periods longer than 6 h (up to 24 h), although during this period the thymidine labelling index dropped from 45% (6 h) to 1% (24 h) (Figure 3a). This finding suggests that in-

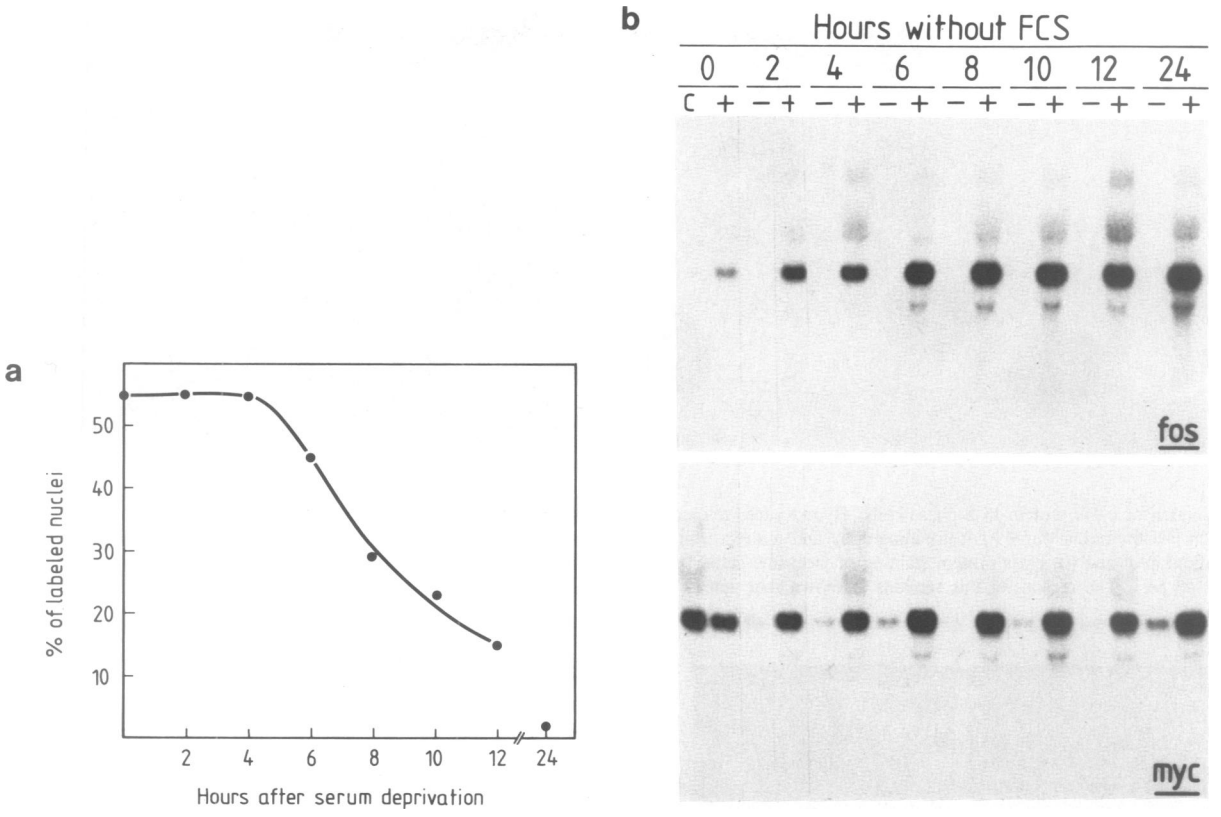

Fig. 3. Induction of c-*fos* and c-*myc* in serum-deprived cells. **(a)** Thymidine labelling index of exponentially growing cells following serum deprivation. Cells were labelled for 2 h every other hour and processed for autoradiography. **(b)** RNA blot analysis of c-*fos* and c-*myc* following serum deprivation (−) in growing cells. At each time indicated after serum deprivation cells were induced with 10% FCS for 1 h (+). C, growing cells prior to serum deprivation.

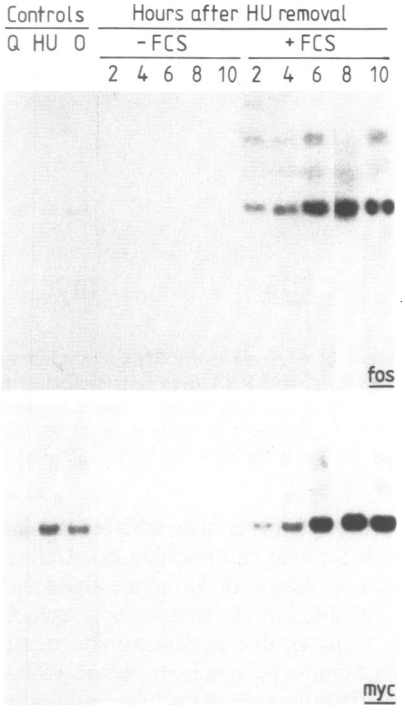

Fig. 4. c-*fos* and c-*myc* induction during S-phase. Hydroxyurea-synchronized cells were released from the block and incubated in serum-free medium for the indicated times. Cells were then either stimulated for 1 h with 10% FCS (+FCS) or not stimulated (−FCS), and the RNA was analyzed for proto-oncogene expression. Q, quiescent cells; HU, quiescent cells 18 h after stimulation in the presence of hydroxyurea; O, after hydroxyurea removal.

ducibility of c-*fos* and c-*myc* is not restricted to quiescent cells. Another interesting observation is that the levels of expression of c-*myc* decreased dramatically after 2 h of serum deprivation, suggesting that its expression requires the constant presence of growth factors in the medium.

c-fos induction during S-phase and other stages of the cell cycle

To obtain direct evidence that c-*fos* expression is inducible in phases other than G0, we decided to study the induction of this proto-oncogene in S-phase populations. For this purpose, quiescent cells were stimulated with 10% FCS, and after 8 h hydroxyurea was added for 6−8 h to block cells at the G1/S boundary. Hydroxyurea was removed, and cells were allowed to proceed through the S-phase in serum-free medium. To determine the inducibility of c-*fos* after hydroxyurea removal, cells were stimulated with 10% FCS every other hour and RNA was analyzed after 1 h of stimulation. Figure 4 demonstrates that maximal induction by FCS was observed at 6−10 h after hydroxyurea removal. At 6 h after hydroxyurea removal the cells were at the peak of the S-phase with a thymidine labelling index of >85% (Figure 2a and data not shown). As illustrated in Figure 4, very similar results were obtained with c-*myc*. Likewise, partially purified FGF, PDGF, and the calcium ionophore A23187 induced both proto-oncogenes.

To prove unequivocally that c-*fos* is inducible during the S-phase, autoradiography and immunofluorescence analyses were carried out on the same cells. Cells synchronized in G1/S by hydroxyurea treatment were released in DMEM without serum. Five hours later, 10% FCS was added together with [^{3}H]thymidine and cells were incubated for 2 h before being processed for immunofluorescence and autoradiography. Figure 5

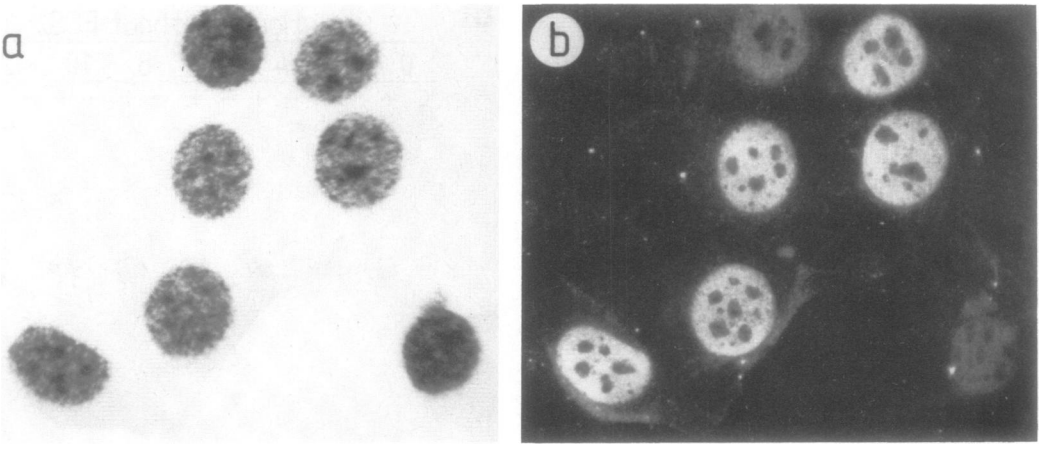

Fig. 5. Induction of c-*fos* protein in S-phase cells. Hydroxyurea-released cells incubated for 5 h in serum-free media were stimulated with 10% FCS and labelled with [^{3}H]thymidine for 2 h. Autoradiography and indirect immunofluorescence were carried out as described in Materials and methods. (**a**) Autoradiography and (**b**) c-*fos* protein staining of the same cells. Immunofluorescent staining of the cells was not uniform (see, for instance, the cell at the lower right corner). The reason for this remains unknown, but generally staining intensity was neither directly nor inversely correlated with thymidine incorporation.

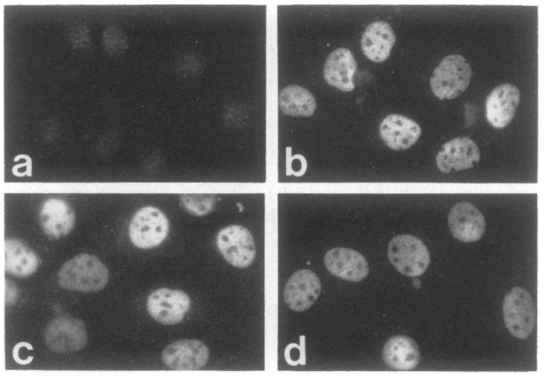

Fig. 6. Induction of c-*fos* protein in G1 and G2 cells. Indirect immunofluorescence was carried out as described in Materials and methods. (**a**) Quiescent cells; (**b**) quiescent cells stimulated with PDGF for 2 h; (**c**) G1 cells treated with PDGF for 2 h, 6 h after release from the quiescent state. In this case quiescent cells were made competent with PDGF for 1 h and then left to progress through G1 in PPP. (**d**) G2 cells treated with PDGF for 2 h. Cells were synchronized by incubating hydroxyurea-released cells in PPP for 9 h. At this time 80–85% of the cells are in G2. In all cases 100 ng/ml of PDGF were used.

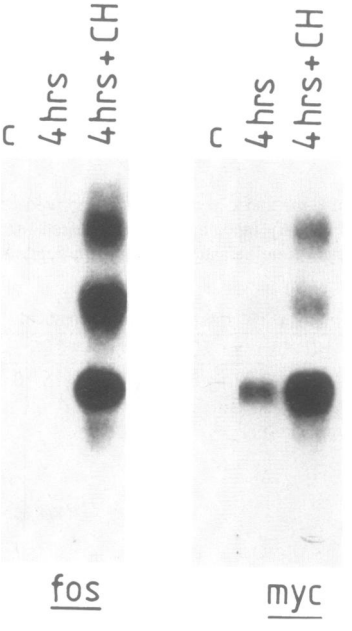

Fig. 7. Superinduction of c-*fos* and c-*myc* RNA by cycloheximide. **c**, RNA blot analysis of c-*fos* and c-*myc* RNA from cells incubated for 4 h in serum-free medium after hydroxyurea removal; **4 h**, cells as in (**c**) but stimulated with FCS for another 4 h; **4 h + CH**, cells as in (**c**) but stimulated with 10% FCS for a further 4 h in the presence of 5×10^{-5} M cycloheximide.

shows clearly that cells engaged in DNA synthesis can be induced to synthesize c-*fos* protein. At the time of induction cells were at the peak of the S-phase and >90% of these cells were replicating DNA. Partially purified growth factors like FGF, PDGF and mitogens like A23187 and the tumor promoter TPA gave similar results (data not shown).

We also analyzed the inducibility of c-*fos* in other phases of the cell cycle. The c-*fos* protein was induced to high levels when cells in G1 or G2 were treated with PDGF for 2 h as illustrated in Figure 6. In contrast, no induction was observed when mitotic cells were treated with either PDGF, FGF, TPA or A23187 (data not shown).

Superinduction of c-fos and c-myc by cycloheximide in S-phase cells

In quiescent NIH3T3 cells c-*fos* and c-*myc* can be superinduced by cycloheximide when added together with growth factors (Cochran *et al.*, 1984; Kelly *et al.*, 1983; Müller *et al.*, 1984). We therefore decided to investigate whether similar results could be obtained with S-phase cell populations. Cells released from hydroxyurea and incubated for 5 h in the absence of serum were induced with 10% FCS in the absence or presence of cycloheximide. Figure 7 shows that cycloheximide treatment led to a superinduction of both c-*fos* and c-*myc* when added together with FCS. While in the absence of cycloheximide c-*myc* and c-*fos* mRNA were found at reduced (c-*myc*) or undetectable (c-*fos*) concentrations 4 h after stimulation, treatment with cycloheximide kept the expression of both proto-oncogenes at very high levels during the same period of time (Figure 7). It thus appears that the c-*fos* gene is subjected to similar regulatory mechanisms in both quiescent and S-phase cells.

Discussion

In this study we have shown that c-*fos* mRNA levels are extremely low or even undetectable throughout the cell cycle in NIH3T3 cells. This suggests that a high expression of c-*fos* is not part of the normal NIH3T3 cell cycle, and thus not required for the continuous cycling of the cells. A role for c-*fos* in the normal proliferation of NIH3T3 cells, however, cannot be completely ruled out, since c-*fos* expression in growing cells has been shown to be slightly elevated compared with quiescent cells (Müller *et al.*, 1984). In contrast, the c-*myc* gene product seems to play a role during the normal proliferation of fibroblasts. First, c-*myc* expression is high in growing, asynchronous cells (Kelly *et al.*, 1983; Campisi *et al.*, 1984; Müller *et al.*, 1984). Second, c-*myc* RNA and protein is readily detectable at all stages of the cell cycle (Hann *et al.*, 1985; Rabbitts *et al.*, 1985; Thompson *et al.*, 1985). The level of expression is, however, lower than in growth factor-stimulated fibroblasts. It appears that the dramatic induction of c-*fos* and c-*myc* by peptide growth factors may be associated with a function that is not part of the normal cell cycle.

Another set of results presented in this study indicates that cells at many stages, and possibly in any phase of the cell cycle other than mitosis, are able to respond to growth factors by inducing c-*fos* and c-*myc* expression, most clearly demonstrated by the induction of both proto-oncogenes in S-phase populations. In the case of the c-*fos* gene, several lines of evidence suggest a role for its encoded product in competence induction in fibroblasts. First, c-*fos* is specifically induced by competence factors, not by the progression factors in PPP (Bravo *et al.*, 1985). Second, mouse fibroblasts can be made competent not only by treatment with certain peptide growth factors, but also by wounding a confluent monolayer of cells (Stiles *et al.*, 1979). Scratching a line into a confluent monolayer of NIH3T3 cells is rapidly followed by the transient induction of c-*fos* protein specifically in cells lining the wound (Müller *et al.*, 1986). Third, the induction of competence is possible at different stages of the cell cycle. Accordingly, we were able to show in this study that c-*fos* is inducible throughout the cell cycle. On the other hand, NIH3T3 cells transformed by *fos* oncogenes are not able to grow in PPP (R.Bravo and R.Müller, unpublished observations), showing that they are not competent. Therefore, if the hypothesis that c-*fos* plays a role in the induction of competence in fibroblasts is correct, then c-*fos* must act in concert with other gene products. It has previously been shown that c-*myc* induces a competence-like state in fibroblasts, but the cells do not become fully independent of competence factors for growth (Armelin *et al.*, 1984; Kaczmarek *et al.*, 1985). It is therefore possible that c-*fos* and c-*myc* cooperate (probably with other unidentified products) in the induction of competence.

In conclusion, we have shown that the c-*fos* gene is expressed at extremely low levels, if at all, during the normal cell cycle, suggesting that its strong induction following growth factor stimulation plays a role only under special circumstances. All available evidence is compatible with the hypothesis that the induction of c-*fos* and c-*myc* may be involved in conferring competence on fibroblasts. In this way, both genes may contribute to the movement of quiescent cells from G0 to G1. It is thus possible that not only c-*myc*, but also c-*fos* induction to high levels is a phenomenon that normally occurs during the recruitment of quiescent cells *in vivo*, such as during wound healing and tissue regeneration (Makino *et al.*, 1984).

Materials and methods

Cell culture

Mouse NIH3T3 cells (clone 7, obtained from D.Lowy, NIH) were grown in DMEM supplemented with 10% FCS and antibiotics (100 units penicillin/ml, 50 μg streptomycin/ml).

Synchronized cell populations

G0 cells. Cells were plated in 130 cm^2 dishes containing 10 ml of DMEM supplemented with 10% FCS. When cultures were ~70% confluent the medium was replaced with DMEM containing 0.5% FCS. After 2–3 days >98% of the cells were quiescent as shown by autoradiography.

G1/S cells. Quiescent cells were stimulated with 10% FCS for 8 h. Then 2 mM hydroxyurea (Sigma) were added to the medium and the cultures incubated for another 8 h, giving a synchronous population of cells in the G1/S boundary (Adams and Lindsay, 1976; Bravo and Macdonald-Bravo, 1985). After removal of the drug, >90% of the cells synchronously entered the S-phase as determined by autoradiography.

Mitotic cells. G1/S populations grown in 175 cm^2 flasks containing 40 ml of DMEM supplemented with 10% FCS were treated with 30 ng nocodazole/ml (Janssen Pharmaceutica) for 1 h, 10 h after hydroxurea removal. Prior to the addition of nocodazole 20 ml of medium from each flask was saved to use it for plating the mitotic cells. Mitotic cells were shaken off and plated in 130 cm^2 dishes.

Analysis of RNA

Isolation and blot analysis of RNA was carried out as described (Müller *et al.*, 1984). Mouse c-*myc* (Sheng-Ong *et al.*, 1982) and c-*fos* (2.45-kb *BglI–SalI* fragment) (Van Beveren *et al.*, 1983) were used as oncogene-specific probes.

Immunofluorescence

For immunofluorescence, cells were fixed with 3% paraformaldehyde, permeabilised with 1% Triton X-100, and stained with a *fos*-specific tumour-bearing rat serum (TBRS) and rhodamine-conjugated goat anti-rat IgG as previously described (Curran *et al.*, 1984).

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