make a better calendrical marker than the midmorning shafts, but not much better.

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27 July 1984; accepted 17 April 1985

Microinjected c-*myc* as a Competence Factor

Abstract. *While a number of oncogenes are expressed in a cell cycle–dependent manner, their role in the control of cell proliferation can only be established by a direct functional assay. The* c-myc *protein, upon microinjection into nuclei of quiescent Swiss 3T3 cells, cooperated with platelet-poor plasma in the stimulation of cellular DNA synthesis. This suggests that* c-myc *protein, like platelet-derived growth factor (PDGF), may act as a competence factor in the cell cycle to promote the progression of cells to S phase. The presence in the medium of an antibody against PDGF abolished DNA synthesis induced by microinjected PDGF; however, the microinjected* c-myc *protein stimulated DNA synthesis even when its own antibody was present in the medium. The* c-myc *protein may act as an intracellular competence factor, while PDGF expresses its biological activity only from outside the cells.*

Microinjection of the cloned Harvey *ras* gene or the $p21^{v\text{-}ras}$ protein induces cellular DNA synthesis in quiescent mammalian cells (*1*, *2*). In contrast, microinjection of a cloned v-*myc* gene (*2*) or of the c-*myc* protein, under the same conditions as those used for the Harvey *ras* gene or protein, fails to induce cellular DNA synthesis in quiescent cells. Nevertheless, a role of c-*myc* in the control of cell proliferation is suggested by several findings including the following: (i) c-*myc* expression is induced by growth factors and mitogens (*3*); (ii) regulation of expression of c-*myc* is altered in chemically transformed mouse cells (*4*); (iii) DNA synthesis occurs when a c-*myc* gene (placed under the control of the mouse mammary tumor virus promoter) is introduced into 3T3 cells in the presence of glucocorticoids (*5*); and (iv) c-*myc* expression is altered in certain forms of neoplasia (*6*).

We now show that microinjected c-*myc* protein induces DNA synthesis in quiescent 3T3 cells if the cells are exposed to platelet-poor plasma (PPP) after microinjection. In the competence-progression model (*7*) c-*myc* can, therefore, be classified as a competence factor. This model is based on the observation that platelet-derived growth factor (PDGF), by itself, cannot stimulate DNA synthesis in quiescent BALB/c 3T3 cells. However, DNA synthesis occurs if cells are treated with PPP after exposure to PDGF. PPP, by itself, has no effect. PDGF makes the cells "competent" (competence factor), and PPP allows their progression into S phase (progression factor).

The purified c-*myc* protein was obtained by recombinant DNA technology as described (*8*). It is recognized by antibodies raised against specific c-*myc* peptides, and, when microinjected into the cytoplasm of cells, rapidly migrates to the nucleus (*9*). In the experiments described below, the c-*myc* protein was microinjected into the nuclei of quiescent cells by means of the manual technique of Graessmann and Graessmann (*10*). For microinjection, the c-*myc* protein and the control protein (albumin) were dissolved in phosphate-buffered saline (PBS) lacking Ca^{2+} and Mg^{2+}, since these two ions can be considered as competence factors. PPP was prepared as described (*11*) from human plasma after removal of platelets and chromatography on CM-cellulose.

Swiss 3T3 cells were plated on glass cover slips in 60-mm petri dishes at a concentration of 5×10^4 per dish in 10 percent fetal calf serum. After 3 days of incubation, the serum concentration was reduced to 1 percent calf serum and incubation continued for 3 to 4 days. At this time, the cells were quiescent and could be used for microinjection. The cells in a circle delimited by etching were microinjected, while the cells outside the circle were used as uninjected controls (Fig. 1). Several experiments were carried out in a similar way with c-*myc* protein or bovine serum albumin, with or without PPP (Fig. 2). Microinjection of the c-*myc* protein (not followed by PPP) caused a small increase in the percentage of cells labeled by [^{3}H]thymidine; an average of 3.9 percent were labeled in the microinjected group as compared to an average of 1.4 percent in the uninjected cells. The significance of this increase is questionable, not only statistically but also biologically. In six separate experiments, in which the cells were treated with PPP (5 percent) after microinjection, c-*myc* protein caused a marked increase in the percentage of cells entering S phase. The average percentage of cells in S phase was 19.7 (range, 10.5 to 30.0 percent) in microinjected cells as compared to an average of 1.5 percent (range, 0.6 to 4.9 percent) in controls. The extent of stimulation is considerable, since, under these conditions (labeling for 24 to 28 hours), serum stimulation of quiescent cells results in the labeling of only about 40 percent of the cells. The possibility that a combination of microinjection and PPP could result in the stimulation of celluar DNA synthesis is ruled out by the experiments in Fig. 2, where c-*myc* protein was replaced by bovine serum albumin. An average of 3.7 percent of cells microinjected with albumin were labeled by [^{3}H]thymidine as compared to 2.1 percent of control cells. We have microinjected a great number of proteins into quiescent 3T3 cells. None stimulated, except c-*ras* (*2*) and the SV40 large T antigen, both of which stimulate cell DNA synthesis even in the absence of PPP.

Our results suggest that c-*myc* is a competence factor, in the competence-

progression model of G_0-S transition (*7, 12*). Other competence factors include fibroblast growth factor (FGF), PDGF, $Ca_3(PO_4)_2$, human serum, SV40, and local wounding of cultured quiescent fibroblast monolayers (*12*). Our findings are in accord with the observation that PDGF induces c-*myc* expression (*3*) and that quiescent cells can be made competent by a c-*myc* gene under the control of the murine mammary tumor virus promoter (*5*).

We then asked whether microinjected PDGF could itself induce cellular DNA synthesis (Table 1). PDGF, microinjected into the cytoplasm, induced cell DNA synthesis when the cells were treated with PPP. However, if an antibody to PDGF was added to the medium surrounding the microinjected cells, the stimulation was abolished. The stimulatory effect of c-*myc* was not affected by addition of an antibody against c-*myc* (*8*) to the culture medium. Thus, PDGF and c-*myc* have similar effects on DNA synthesis, but c-*myc* is effective in the interior of the cell and PDGF acts on the cell surface. The PDGF microinjected into the cytoplasm probably leaked out slowly as suggested by the high percentage of labeled control cells around the area of cells microinjected with PDGF. Moreover, this high percentage decreased sharply at greater distances from the microinjected area. Coinjection of c-*myc* and its antibody is not feasible, because the fine precipitate clogs the tiny openings of the micropipette. We did not attempt to separately microinject a protein and its antibody in the same cell, because our experience has shown that two microinjections cause too many cells to die.

Table 1. The effect of an extracellular antibody against PDGF or c-*myc* protein on the stimulation of DNA synthesis in quiescent cells cultured in the presence of PPP. PDGF (120 unit/ml) was microinjected into the cytoplasm; c-*myc* (1 mg/ml) was microinjected into the nucleus. Results are the average from three independent experiments at each point. Antibodies were added after microinjection at a concentration of 20 μg/ml for the polyclonal antibody against PDGF and 2 μg/ml for the monoclonal antibody against c-*myc* protein. Percentage of labeled cells was measured 26 hours after microinjection. M, microinjected; C, uninjected.

Microinjected protein	Labeled cells in the absence of specific antibody (%)		Labeled cells cultured in the presence of specific antibody (%)	
	M	C	M	C
PDGF	60.0	36.0	0.9	0.9
c-*myc*	30.0	1.5	30.0	0.8

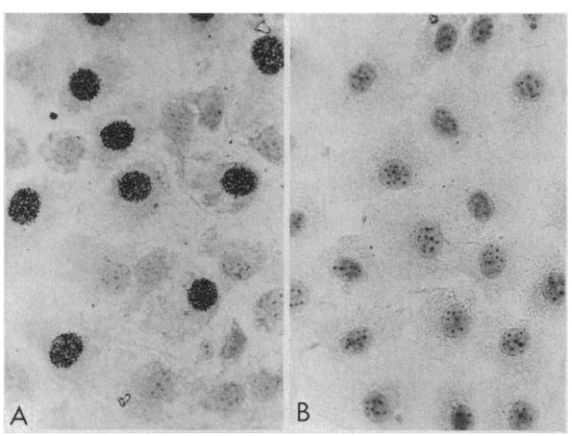

Fig. 1. (A) Photomicrograph of Swiss 3T3 cells microinjected with c-*myc* protein (1 milligram per milliliter of PBS without Ca^{2+} and Mg^{2+}) and uninjected (B). Cells were made quiescent, microinjected, and then grown in 5 percent PPP and [^{3}H]thymidine (0.2 μCi/ml) for 28 hours. After this time, cells were processed for autoradiography and stained with Giemsa. Both panels show cells from the same cover slip, with cells treated in exactly the same way except for microinjection.

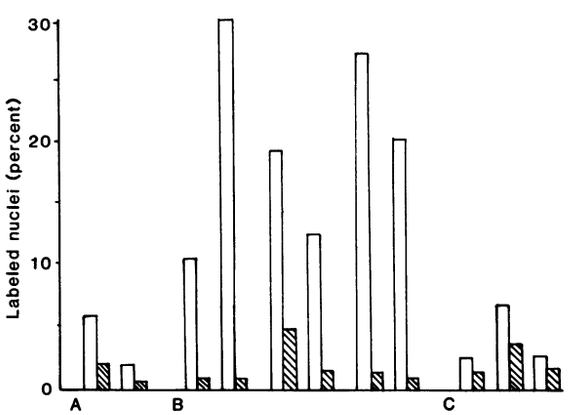

Fig. 2. Percentages of labeled nuclei from microinjected and uninjected cells from the same cover slips. Cells were grown and processed as described in the legend to Fig. 1. Each pair of bars represents an independent experiment. (A) Cells microinjected with c-*myc* protein (open bars) and control cells (shaded bars), cultured in the absence of PPP. (B) Cells microinjected with c-*myc* protein (open bars) and control cells (shaded bars), cultured with 5 percent PPP. (C) Cells microinjected with bovine serum albumin (open bars) (1 mg/ml) and uninjected cells (shaded bars) cultured in the presence of 5 percent PPP. All cells in the microinjected circles were counted (between 80 and 120 cells per experiment), and at least 500 uninjected cells.

In conclusion, our experiments show that c-*myc* can act as a competence factor in stimulating cell proliferation as already suggested by Stiles and co-workers (*5, 13*). Our experiments point out the separate roles of c-*myc* and PPP. These results are compatible with the observation that the level of expression of c-*myc* is often high in proliferating cells (*3–6*) and is sometimes elevated in nonproliferating tissues (*14*). If c-*myc* is a competence factor, its presence in the absence of a progression factor would not result in cell proliferation. The competence-progression theory can be generalized into a model in which cell cycle progression depends on the successive appearance of different growth factors, receptors for growth factors, and internal regulatory proteins (*15*). Our results agree with the report (*16*) that the transforming protein of v-*sis*, which is highly homologous to PDGF (*17*), acts as an autocrine factor through surface receptors. The expression of certain genes is induced by PDGF (*18*) or by serum, which contains PDGF (*19*). It would be interesting to know whether c-*myc* activates gene expression. If so, the same set of genes might be activated by c-*myc* as PDGF or they might constitute a subset of those activated by PDGF. Furthermore, the number of genes activated by c-*myc* may be less than that induced by v-*ras*, which can stimulate cellular DNA synthesis in the absence of PPP.

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20. We thank G. Rovera for the gift of one of the antibodies against c-*myc*, W. Niewiarowski for the antibody to PDGF, J. W. Pledger for the PDGF, and A. Jones for technical assistance. Supported by a grant (CD 214) from the American Cancer Society.

26 December 1984; accepted 4 March 1985

Filamentous Fusion Phage: Novel Expression Vectors That Display Cloned Antigens on the Virion Surface

Abstract. *Foreign DNA fragments can be inserted into filamentous phage gene III to create a fusion protein with the foreign sequence in the middle. The fusion protein is incorporated into the virion, which retains infectivity and displays the foreign amino acids in immunologically accessible form. These "fusion phage" can be enriched more than 1000-fold over ordinary phage by affinity for antibody directed against the foreign sequence. Fusion phage may provide a simple way of cloning a gene when an antibody against the product of that gene is available.*

Gene III of filamentous phage encodes a minor coat protein, pIII, located at one end of the virion (*1, 2*). The amino-terminal half of pIII binds to the F pilus during infection, while the carboxyl-terminal half is buried in the virion and participates in morphogenesis (*3, 4*). I report here that a foreign sequence can be inserted between the two domains without unduly disrupting pIII function and that the foreign sequence is displayed in immunologically accessible form on the infectious particle. Affinity purification of such "fusion phage" may provide a way of cloning a gene from a library of random inserts when antibody against the gene product is available; this scheme could be simpler than present methods, in which antibody is used indirectly to screen a conventional expression-vector library for the desired clones (*5*).

As a source of model inserts I used a Sau 3A digest of plasmid pAN4, which encodes the Eco RI endonuclease and methylase enzymes (*6*); the gene III insertion site was the unique Bam HI site of phage f1 (*7*). Several pAN4 fragments were candidate inserts, in that they would preserve the gene III reading frame without stop codons when ligated into the Bam HI site; the other fragments would result in noninfective particles. Two candidate inserts derive from the endonuclease gene: a 132-base-pair (bp) fragment (nucleotides 416 to 547) and a 171-bp fragment (nucleotides 548 to 718) (*6*); a mutant pIII with these inserts would bear a segment of endonuclease sequence.

Accordingly, a Sau 3A digest of pAN4 was ligated with Bam HI–cleaved f1 DNA and transfected into *Escherichia coli* strain K38 (legend to Fig. 1) (*8*). Twenty-three of the 24 clones examined could not be cleaved by Bam HI and thus were presumed to harbor gene III inserts. By restriction analysis at least three different inserts could be distinguished, including the 171-bp (but not the 132-bp) endonuclease fragment.

One clone with the 171-bp insert, fECO1, was propagated and studied further. Antibody to Eco RI endonuclease (*9*) markedly reduced the fECO1 titer, but it had no effect on the f1 parent; the phage-neutralizing activity of the antibody could be completely blocked by preincubation with purified endonuclease (Fig. 1) (*10*). This demonstrates that antigenic determinants coded by foreign inserts can be displayed on the virion surface. Buffer at *p*H 2.2 or 2.7 reversed the reaction of phage with antibody, restoring most or all of the original titer.

Phage fECO1 gave very small plaques on strain K38 and almost invisible plaques on strain JM107 (*11*). The stationary phase titer was 100 times lower than normal; however, the number of physical particles (quantified spectrophotometrically) (*12*) exceeded the number of plaque-forming units (PFU) by a factor of more than 50, compared with a factor of about 2 for wild-type f1. These results indicate that the phage are partially defective in infectivity. They also show a considerably higher incidence of multimeric "polyphage" (*1*) than normal, as determined by whole-virion electrophoresis (*13*) and electron microscopy. Neither abnormality is surprising in view of pIII's role in infection and morphogenesis (*3, 4*). These defects probably put fusion phage at a selective disadvantage with respect to variants in which the insert has been lost or altered. Indeed, one attempt at propagating fECO1 produced phage with an apparently altered or deleted insert, as they could not be detectably neutralized with antibody to endonuclease. The accumulation of unwanted variants might be circumvented by using fd-tet as the vector, since it can be propagated as a plasmid, independently of gene III function (*4*).

In the following experiment I tested the ability of antibody to affinity-purify cognate fusion phage from a large background of noncognate phage, such as would be present in a fusion phage library. Antibody was adsorbed to a small polystyrene petri dish, the remaining adsorption sites being blocked with excess protein. A suspension containing fECO1 phage and a large excess of the double-amber mutant strain M13mp8 (*14*) was swirled in the dish, which was then washed extensively with fresh medium. The dish was eluted with acidic buffer, and the fECO1 and M13mp8 phage in the eluate were separately titered.

As shown in Table 1, fECO1 was enriched over M13mp8 by factors of 1500 to 7200 in the three experiments. Detailed analysis of the results of experiment 3 suggested two ways in which purification might be improved (Table 1). First, there were appreciable M13mp8 phage but few fECO1 phage in the last three washes, suggesting that further washing might be useful. Second, there were considerably more M13mp8 phage