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Oncogenic v-Abl tyrosine kinase can inhibit or stimulate growth, depending on the cell context

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The v-*abl* oncogene of Abelson murine leukemia virus (A-MuLV) induces two opposite phenotypes in NIH3T3 cells. In the majority of cells, v-*abl* causes a growth arrest at the G_1 phase of the cell cycle; while in a minority of cells, v-*abl* abrogates the requirement for growth factors. Using temperature sensitive mutants, it can be demonstrated that v-Abl tyrosine kinase is required for growth inhibition or stimulation. The two phenotypes are not caused by mutations or differences in the expression of v-Abl, but are dependent on the cell context. Two stable subclones of NIH3T3 cells have been isolated that exhibit similar morphology and growth characteristics. However, upon infection with A-MuLV, the 'positive' cells become serum- and anchorage-independent, whereas the 'negative' cells become arrested in G_1. The positive phenotype is dominant, shown by cell fusion, and treatment with 5-azacytidine converts the negative cells to the positive phenotype. Activation of v-Abl tyrosine kinase induces the serum-responsive genes in the positive but not in the negative cells. Transactivation of the c-*fos* promoter by v-Abl in transient assays is also restricted to the positive cells. These results show that v-Abl tyrosine kinase is not an obligatory activator of growth, but requires a permissive cellular context to manifest its mitogenic function.

Key words: 5-azacytidine/cell cycle/growth factor/*fos* promoter/PI kinase

Introduction

Many growth factors activate protein tyrosine kinases that are part of or associated with the receptors to transmit the mitogenic signals (Rudd, 1990; Ullrich and Schlessinger, 1990). While controlled activation of tyrosine kinases promotes normal proliferation, deregulated tyrosine kinases can cause neoplastic transformation. Oncogenic tyrosine kinases are likely to transform cells by the continuous activation of the mitogenic program (Bishop, 1987). If the oncogenic tyrosine kinases activate the mitogenic program continuously, they should be able to abrogate the growth factor requirement of every cell. This, however, is not true for the tyrosine kinase encoded by the v-*abl* oncogene of the Abelson murine leukemia virus (A-MuLV).

There has been much evidence indicating that the v-Abl tyrosine kinase can stimulate cell proliferation. A-MuLV induces pre-B lymphomas in mice and transforms lymphoid, myeloid and fibroblastic cells *in vitro* (for a recent update, see Risser and Green, 1988). NIH3T3 cells transformed by A-MuLV can grow without serum (Rees-Jones *et al.*, 1989) and hematopoietic cells grow without interleukin-3 (Cook *et al.*, 1985; Pierce *et al.*, 1985) or interleukin-2 (Cook *et al.*, 1987). The abrogation of growth factor requirement in A-MuLV transformed cells can occur through a non-autocrine mechanism, in which the v-Abl tyrosine kinase is essential for the maintenance of factor-independent proliferation (Kipreos and Wang, 1988). Several observations suggest that the v-Abl tyrosine kinase can act on the mitogenic pathway. Phosphotidylinositol 3-kinase (PI 3-kinase), an enzyme shown to be associated with the activated receptors of several growth factors (Cantley *et al.*, 1991), is found phosphorylated on tyrosine and physically associated with v-Abl in A-MuLV transformed cells (Varticovski *et al.*, 1991). Activation of the v-Abl tyrosine kinase also induces the c-*myc* gene in A-MuLV transformed myeloid cells (Cleveland *et al.*, 1989). While these findings suggest that v-Abl can activate the mitogenic program, it is not clear how the mitogenic activity of v-Abl can induce neoplastic transformation.

The v-*abl* oncogene has also been reported to have a 'lethal' effect on fibroblasts. Balb/c3T3 cells are resistant to transformation by A-MuLV and the few Balb/c foci obtained were found to express mutated forms of the v-Abl protein, indicating that the wild type v-Abl might inhibit cell growth (Ziegler *et al.*, 1981). When NIH3T3 cells were transfected with the A-MuLV DNA, very few foci were obtained (Goff *et al.*, 1982). This low efficiency was thought to be due to a lethal effect of the A-MuLV DNA because that DNA could suppress the formation of mycophenolic acid-resistant colonies when co-transfected with the *gpt* (xanthine-guanine phosphoribosyltransferase) gene (Goff *et al.*, 1982). Since cell death was not directly demonstrated in those reports, an arrest of cell growth by the v-*abl* oncogene could also have accounted for the observations.

Although A-MuLV induces foci in NIH3T3 cells with a high efficiency, most of those foci are small and cannot be established into transformed lines. When the A-MuLV induced foci were picked and replated, morphologically transformed cells quickly disappeared. Only a few of the very large foci could grow as transformed cell lines. Since the existing A-MuLV transformed lines have been established from a few of the foci, their properties may not be representative of all cells expressing the v-*abl* oncogene. In other words, the primary biological effect of the v-Abl tyrosine kinase might not be recognized in cell lines that were selected for transformation.

To establish A-MuLV infected NIH3T3 cells without selecting for transformation, we used two temperature sensitive A-MuLV mutants (Kipreos *et al.*, 1987). The mutant A-MuLV contained the DP or RK v-*abl* gene, which was selected in bacteria to encode temperature sensitive

tyrosine kinases (Kipreos *et al.*, 1987). Both mutant kinases are inactivated at 39°C (Kipreos *et al.*, 1987). The mutant kinases are reactivated within 30 min after shifting the temperature to 32°C, even in the absence of new protein synthesis (E.T.Kipreos, unpublished observations). When the mutant v-*abl* genes were incorporated into A-MuLV, temperature sensitive transforming viruses were obtained (Kipreos *et al.*, 1987; Kipreos and Wang, 1988). With these conditional mutants of A-MuLV, it was possible to establish NIH3T3 clones that were infected, but untransformed at 39°C. When the v-Abl tyrosine kinase was activated in those clones, we found that it caused cell cycle arrest. The pursuit of that result has led to the isolation of two distinct NIH3T3 cell types. In the 'negative' NIH3T3 cells, v-Abl tyrosine kinase inhibits cell growth by causing a G_1 arrest. In the 'positive' NIH3T3 cells, however, v-Abl tyrosine kinase abrogates the requirement for growth factors. These results show that transformation by the v-*abl* oncogene requires a permissive cellular context that overrides the growth inhibitory activity of v-Abl and allows v-Abl to activate the mitogenic pathway.

Results

A majority of A-MuLV infected NIH3T3 cells become growth arrested

The growth inhibitory activity of v-*abl* was discovered when cells infected with the temperature sensitive mutant of A-MuLV were clonally established at the restrictive temperature (39°C). NIH3T3 cells were infected with the DP mutant of A-MuLV (Kipreos *et al.*, 1987; Kipreos and Wang, 1988) and individual clones were isolated by limited dilution and propagation at 39°C. Each clone was screened for v-Abl expression by immunoblotting. A dozen v-Abl expressing lines were obtained and they showed normal NIH3T3 phenotypes at 39°C. When shifted to the permissive temperature (32°C), however, these v-Abl expressing clones stopped growing, while uninfected NIH3T3 cells grew normally (Figure 1). Activation of the v-*abl* oncogene did not kill cells because there was no increase in the number of dead cells at 32°C. The growth inhibition was fully reversible after shifting back to 39°C. Since the v-*abl* mutants were selected in bacteria as temperature sensitive kinase mutants (Kipreos *et al.*, 1987), the arrest of cell growth was correlated with the activation of v-Abl tyrosine kinase.

The level of v-Abl varied between 1- and 33-fold more than that of c-Abl among the dozen clones. A clone that expressed an active v-Abl at the same level of c-Abl grew normally at 32°C. To cause growth arrest, the expression of v-Abl had to be at least 3-fold more than that of the endogenous c-Abl. A correlation between the level of v-Abl and the severity of morphological transformation was noticed, the high expressers (>10 times c-Abl) became rounded, while the low expressers were only elongated at 32°C. However, growth arrest was observed with all clones that expressed v-Abl above the threshold level, irrespective of cell shape (data not shown).

The v-Abl-induced growth arrest was cell cycle specific, with a majority of the cells blocked in G_1. This was shown by shifting a synchronous population of cells to 32°C (Figure 2). D4X cells or uninfected NIH3T3 cells were made quiescent at 39°C and then stimulated with serum. At 14 h after the addition of serum, when most cells had entered S

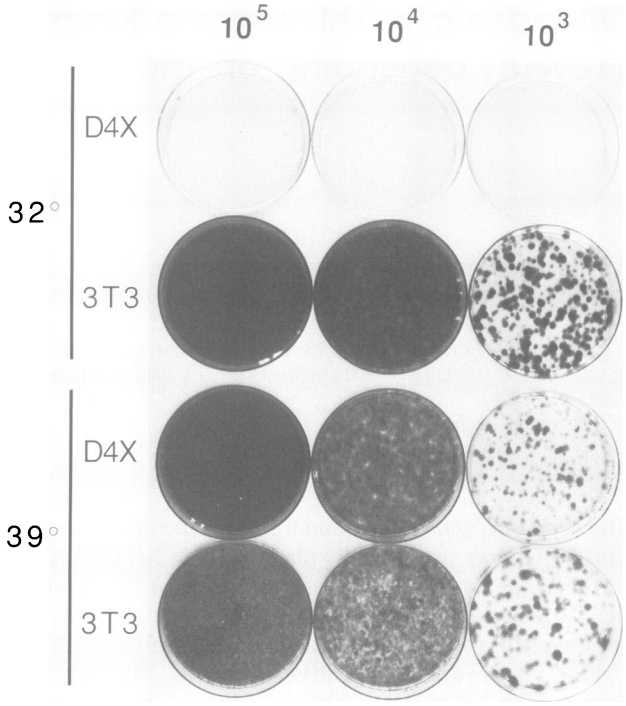

Fig. 1. Growth inhibition by the v-Abl tyrosine kinase. Uninfected NIH3T3 cells or temperature sensitive A-MuLV infected D4X cells were seeded at 1×10^3, 1×10^4 and 1×10^5 cells per 10 cm dish and cultured for either 12 days at 39°C or 24 days at 32°C. Colonies were then stained with a solution of 1% crystal violet in 20% ethanol to visualize cell growth.

phase, the cultures were shifted to 32°C (Figure 2). The uninfected NIH3T3 cells progressed through the cell cycle at 32°C and became unsynchronized with cells in G_1, S and G_2 by 48 h after serum addition (Figure 2). Activation of the v-Abl tyrosine kinase (reactivation occurred within 30 min of temperature shift) did not block the progression through S, G_2 or M as evident by the presence of G_1 and G_2 cells at 30 h. However, entry into the next S phase was blocked (Figure 2, h 38 and 48). In a v-Abl arrested population, between 70 and 72% of the cells were in G_1, 14–16% in S and 14% in G_2. An uninfected NIH3T3 population, on the other hand, was 33–37% G_1, 45–54% S and 13–19% G_2 at 32°C. When the G_1-blocked D4X cells were shifted to 39°C, a burst of incorporation of [^{3}H]thymidine was observed at 10 h after the temperature shift (data not shown). This result indicated that the v-Abl tyrosine kinase blocked cells at an early point in the G_1 phase of the cell cycle.

Growth arrest could be observed with whole populations of A-MuLV infected cells prior to cloning. NIH3T3 cells were infected with the wild type or the temperature sensitive mutant A-MuLVs at a m.o.i. of 10. The whole populations were then analysed for cell cycle distribution at 3 days post-infection. These analyses showed that the wild type A-MuLV caused G_1 arrest (76–80% G_1) at both 32°C and 39°C, whereas the temperature sensitive mutant only induced G_1 arrest at 32°C (70–75% G_1). Mock-infected NIH3T3 cells had a G_1 content of 36–42% in those experiments. Thus, growth arrest is observed with a majority of the A-MuLV infected NIH3T3 cells.

That v-Abl induces growth arrest seems at odds with the

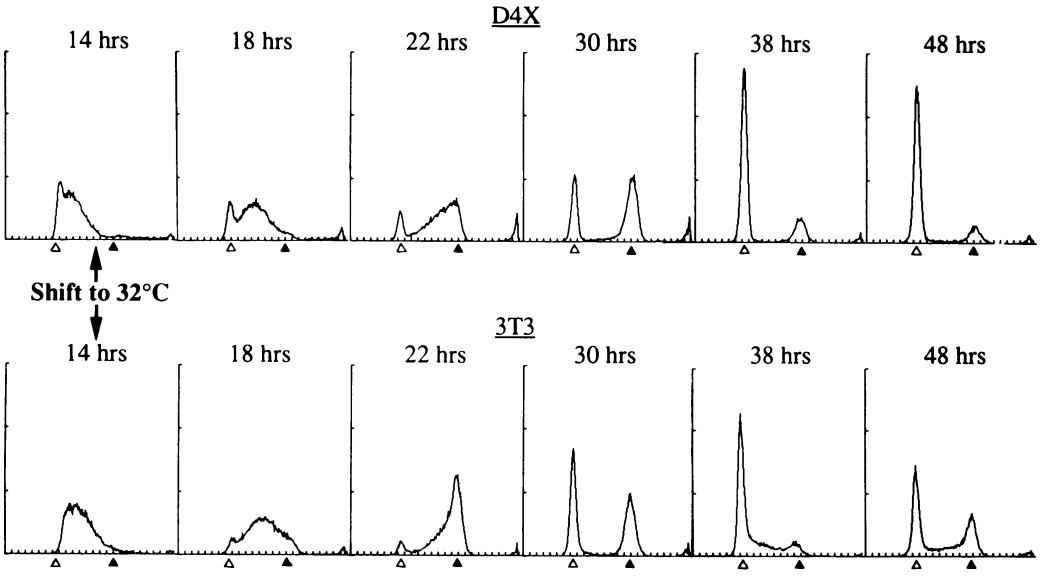

Fig. 2. Cell cycle analysis of the growth arrest induced by v-Abl tyrosine kinase. D4X and uninfected NIH3T3 cells were each seeded in six 10 cm plates, at 1×10^6 cells per plate. Cells were made quiescent by incubating for 48 h in DMEM containing 0.5% serum at 39°C. At time 0, cells were stimulated with 20% serum at 39°C. After 14 h plates were shifted to 32°C. Flow cytometry was performed on cells harvested at the h indicated, post-serum stimulation. Triangles indicate the position of G_1 (open triangles) or G_2/M (closed triangles) content of DNA.

observation that it causes foci formation with a high efficiency. Focal growth of A-MuLV infected cells is usually assayed on a monolayer of uninfected cells. To determine if normal NIH3T3 cells could support the focal growth, D4X cells were co-cultivated with uninfected NIH3T3 cells that had been pretreated with mitomycin C to prevent their growth. Under these co-cultivation conditions, limited focal growth of the D4X cells was observed. However, when plated on plastic dishes alone, D4X cells did not form colonies (Figure 1). Although a monolayer of mitomycin C-treated NIH3T3 cells could support a limited growth, plating on fibronectin could not rescue the D4X cells. Conditioned medium from uninfected or fully transformed NIH3T3 cells (e.g. D5 cells, see below) also failed to support the growth of D4X cells. Mitogenic factors such as phorbol esters or high concentrations of serum did not reverse the growth arrest either. These results indicate that the uninfected cells are required for the formation of foci and explain the difficulty in establishing cell lines from v-Abl-induced foci.

A minority of A-MuLV infected NIH3T3 cells become factor and anchorage-independent for growth

Many transformed lines from A-MuLV infected NIH3T3 cells have been established and they exhibit the classical serum- and anchorage-independent growth phenotypes. Using the temperature sensitive A-MuLV, we have also isolated transformed lines that are temperature sensitive for the transformed phenotypes (Kipreos *et al.*, 1987). Since a majority of A-MuLV infected cells become arrested in G_1, the fully transformed cells must arise from a small number of A-MuLV infected cells. The frequency at which infected cells acquired the serum-independent growth phenotype was determined by a statistical analysis (see Materials and methods). The quantitative analysis demonstrated that on average only 5% of the A-MuLV infected cell acquired the ability to grow in serum-free medium (Materials and methods). By plating a known number of infected cells in soft agar, the frequency of anchorage-independent growth was also found to be 5%. Hence, only a minority of A-MuLV infected NIH3T3 cells become competent to grow in serum-free medium or in soft agar.

With the temperature sensitive A-MuLV mutants, we have established lines that were isolated as soft agar colonies at 32°C, e.g. D5. In these lines, v-Abl tyrosine kinase activity was required to induce and maintain the serum- and anchorage-independent growth. The D4X- and the D5-like cell lines have been maintained over longterm passages without phenotypic conversion. The D4X cells could be maintained at 32°C for weeks without forming colonies in regular medium, serum-free medium or in soft agar, but they grew normally in serum-supplemented medium at 39°C. On the other hand, the D5 cells grew vigorously in regular medium, serum-free medium or in soft agar at 32°C and reverted back to serum and anchorage-dependent growth at 39°C. Taken together, these results show that the v-Abl tyrosine kinase inhibits growth in a majority of NIH3T3 cells but can stimulate growth in a minority of those cells.

Cell type-dependent induction of serum-responsive genes by V-Abl

Because v-Abl tyrosine kinase could stimulate the proliferation of some NIH3T3 cells, we determined whether it could activate the serum-responsive genes, such as c-*fos*, c-*myc* and c-*jun*. Activation of the v-Abl tyrosine kinase in D5 cells by a temperature shift indeed led to the induction of serum-responsive genes (Figure 3). The induction occurred whether the D5 cells were quiescent or exponentially growing, although the level of induction for the c-*fos*, c-*myc* and c-*jun* RNA was lower in growing cells (Figure 3). These results were similar to the effect of the platelet-derived growth factor on those genes in quiescent or growing cells (Bravo *et al.*, 1986; Morgan and Pledger, 1989). Nuclear run-on experiments showed an increase in transcription when v-Abl tyrosine kinase was activated (data not shown). Treatment with cyclohexamide prior to the temperature shift did not block the induction, thus new protein synthesis was not required for v-Abl to activate the serum-responsive genes (data not shown). The induction was

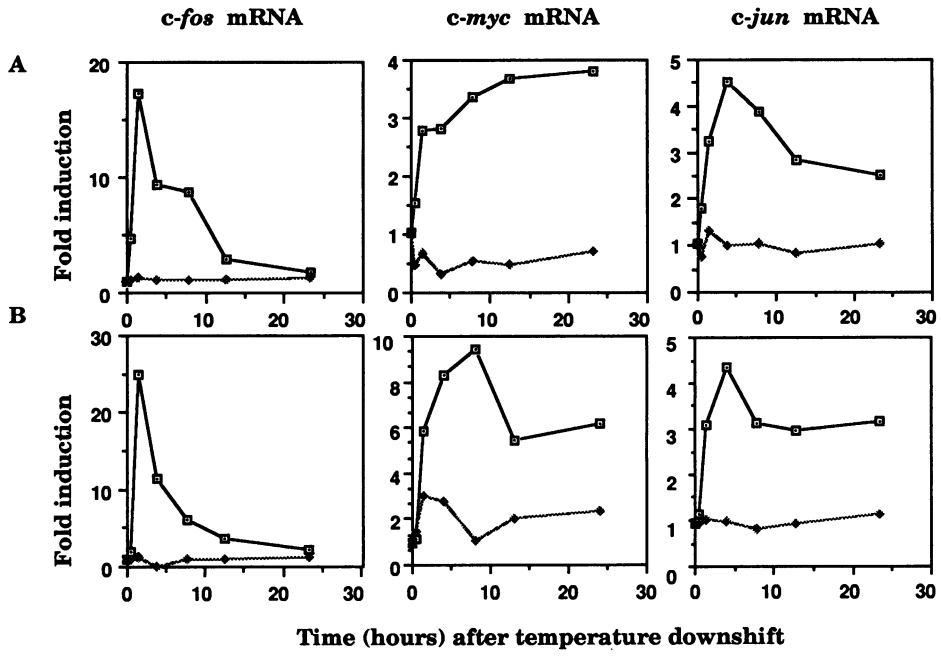

Fig. 3. Cell type-dependent induction of serum-responsive genes by v-Abl tyrosine kinase. The temperature sensitive v-Abl tyrosine kinase was activated by shifting the temperature from 39°C to 32°C. Total RNA was isolated at the indicated times, post-temperature shift, from the v-Abl growth-stimulated clone D5 (open squares) or the v-Abl growth-inhibited clone D4X (filled diamonds). The relative level of c-*fos*, c-*myc* and c-*jun* mRNAs was measured by northern blot analyses; comparing v-Abl induced level to the uninduced level at 39°C (0 h). Values were normalized to β_2 microglobulin levels that were not affected by the temperature shift. **(A)** Induction of serum-responsive genes by v-Abl in exponentially growing cells. Asynchronous D5 and D4X cells growing at 39°C, were shifted to 32°C in 10% serum. **(B)** Induction of serum-responsive genes by v-Abl in quiescent cells. D5 and D4X cells were made quiescent by incubating in serum-free medium at 39°C for 48 h, prior to temperature shifting in serum-free medium.

reduced by 8-fold, but not completely abolished, by the addition of protein kinase inhibitor (H7) or by a prolonged pretreatment with phorbol ester to down regulate protein kinase C (data not shown).

Induction of serum-responsive genes by v-Abl is cell type-dependent. Activation of v-Abl did not induce c-*fos*, c-*myc* or c-*jun* in either quiescent or exponentially growing D4X cells (Figure 3). This was consistent with the fact that v-Abl did not stimulate growth in these cells. We also examined polyclonal populations of temperature sensitive A-MuLV infected cells for the induction of the c-*fos* and the c-*myc* genes. When the temperature shift experiment was performed with a whole population of temperature sensitive A-MuLV infected cells, no induction of c-*fos* or c-*myc* was observed. However, in a pool of polyclonal cells selected as soft agar colonies, the temperature shift led to the induction of c-*fos* and c-*myc* at the same level found in the D5 cells. Therefore the growth stimulating activity of v-Abl is correlated with the induction of serum-responsive genes, but the effect is only found with a minority of A-MuLV infected NIH3T3 cells.

The growth stimulatory phenotype is dominant

To determine which of the two v-*abl*-induced growth phenotypes was dominant, cell fusion experiments were performed. Neomycin or hygromycin resistant clones of the D4X, D5 and uninfected NIH3T3 cells were established and the growth phenotype of each clone was verified (Materials and methods). Three sets of fusions were carried out between D4X/D5, D4X/3T3 and D5/3T3. Double resistance cells were established at 39°C, so as not to select against any phenotype (Materials and methods). The chromosome numbers of the double resistant cells were counted to verify the isolation of hybrids (Table I). The growth phenotype of the hybrids was determined by measuring the rate of [^{3}H]thymidine incorporation in regular or serum-free medium at 39°C and 32°C. The induction of c-Fos protein was determined by immunofluorescent staining of the hybrid cells after shifting to 32°C. The D5/D4X hybrids were found to grow in serum-free medium and they expressed Fos upon temperature shift to 32°C (Table I). D5/3T3 hybrids exhibited the D5 phenotype as well (Table I). The D4X/3T3 hybrids displayed the D4X phenotype of undergoing growth arrest (Table I). This showed that the fusion procedure did not select against the growth inhibition phenotype. These results were obtained with hybrids derived from three independent fusion experiments and they indicated that the growth stimulatory phenotype was dominant.

Phenotypic variation is determined by the context of NIH3T3 cells

The development of a dominant growth stimulatory (GS) phenotype and a recessive growth inhibitory (GI) phenotype in A-MuLV infected cells could be due to one of three possibilities. First, there might be a dominant mutant A-MuLV virus, comprising 5% of our viral stock, which conferred the GS phenotype. A second possibility was that only those NIH3T3 cells that expressed v-*abl* above a critical threshold level could acquire the GS phenotype and this threshold level was achieved in 5% of the infected cells. The third possibility was that the NIH3T3 cells were heterogeneous. A subset of cells comprising 5% of the population expressed a dominant function which was required for v-*abl* to induce the GS phenotype.

Table I. The A-MuLV-induced growth stimulatory phenotype is dominant

Fusion[a]	Chromosome[b]	[^{3}H]thymidine incorporation[c] ($\times 10^3$ c.p.m.)				
		39°C+	39°C−	32°C+	32°C−	c-Fos[d]
D5 Hyg/ D4X Neo	117 ± 11	57.0	1.1	22.0	13.0	+
D5 Hyg/ 3T3 Neo	112 ± 8	18.0	0.3	13.0	5.0	+
D4X Neo/ 3T3 Hyg	114 ± 10	13.0	1.5	0.7	0.2	−

[a]Cell lines bearing selectable antibiotic resistant markers for either neomycin (Neo) or hygromycin (Hyg) were fused. Double antibiotic resistant hybrids were selected at the restrictive temperature (39°C). Several colonies were pooled from each fusion for analyses.
[b]The average number of mitotic chromosomes was determined. Parental lines used for fusion, D5, D4X or 3T3, contained an average of 61 ± 5 chromosomes per cell.
[c]The growth phenotype was determined by measuring the incorporation of [^{3}H]thymidine after a 1 h pulse. Cells were plated at either 39°C or 32°C in 10% serum (+) or serum-free medium (−) for 48 h prior to labelling.
[d]Induction of c-Fos protein by v-Abl was determined by indirect immunofluorescent staining with an anti-Fos antibody. Cells were shifted to 32°C for 45 min and then fixed and stained using previously described methods (De Togni *et al.*, 1988).

If the GS phenotype was caused by a mutant A-MuLV, virus produced by the D5 cells should confer this phenotype at a much higher frequency. This was found not to be true. Virus obtained from D5 cells gave rise to the GS phenotype at the same low frequency as virus produced by the D4X cells. The 5% frequency of the GS phenotype was determined with a m.o.i. of 0.01. Should there be a dominant mutant virus present at 5%, the frequency ought to increase at higher m.o.i. when each cell was infected with more than one virus. At an m.o.i. of 10, theoretical calculations with the Poisson distribution equation gave an expected frequency of 39% for the GS phenotype, assuming that the mutant virus and the wild type virus infected cells equally well (Materials and methods). When the experiment was performed, higher levels of v-Abl expression were observed with higher m.o.i.s infections; however, the same 5% average frequency was obtained with m.o.i. between 0.01 and 40. Since the frequency of the GS phenotype was not affected by m.o.i., it did not support the mutant virus theory.

If the GS phenotype required a higher level of v-Abl, then the GS clones should contain on average more v-Abl than the GI clones. The average level of v-Abl and of the tyrosine phosphorylated proteins were determined by quantitative immunoblotting in several polyclonal populations of GS and GI clones. The results showed no difference in v-Abl or its tyrosine kinase activity between the pools of GS and the GI clones (data not shown). It thus appeared that the level of v-Abl was also not a determinant of the phenotype.

If the two phenotypes were caused by pre-existing differences among NIH3T3 cells, subclones of NIH3T3 cells should respond to v-Abl either exclusively as a growth stimulator or an inhibitor. A majority of the subclones should exhibit the GI phenotype and a minority the GS phenotype. As mentioned above, infected cells of the GS phenotype form colonies in soft agar. Therefore 94 subclones of NIH3T3 cells were prepared and a portion of each clone was infected with A-MuLV and then plated in soft agar. Eighty-six out of the 94 infections were scored, among them eight clones produced numerous soft agar colonies while 57 clones gave no soft agar colonies after infection with A-MuLV. The other clones gave intermediate numbers of colonies. These were likely to be mixed clones and they were not studied further.

Two representative subclones, 4A2 and 1A4, were kept for further investigation. When infected with A-MuLV, 89% of the 4A2 foci formed colonies in soft agar and 93% in serum free medium. On the other hand, none of the 1A4 foci formed colonies in soft agar or serum-free medium. The successful isolation of these subclones demonstrated that the GS and GI phenotypes arose from pre-existing differences among NIH3T3 cells. The 4A2 clone was called the positive, whereas the 1A4 clone the negative NIH3T3 cells.

Differential response to V-Abl by the two NIH3T3 subclones

The morphology of the uninfected 4A2+ and the 1A4− cells was very similar (Figure 4A). In 10% serum, both doubled every 25 h, except that the saturation density of 4A2+ was about twice that of 1A4− (Figure 4C). When fed daily with 1% serum, both cell types doubled once every 31 h, and they saturated at lower densities than in 10% serum (Figure 4C). When the serum concentration was reduced to 0.33%, 1A4− cells became arrested, whereas 4A2+ cells grew at a low but detectable rate (114 h doubling time) and saturated at an even lower density (Figure 4C). Growth response, determined by [^{3}H]thymidine pulse labelling, as a function of serum concentration was also measured and it showed that the two clones had a similar sensitivity to serum growth factors (Figure 4B). In the low passage NIH3T3 cells used in our study, the positive clones were present at 5−8%. Since the positive cells would have a growth advantage if a heterogeneous population of NIH3T3 cells was cultured at high density or in reduced serum, the frequency of the positive cell type could conceivably be increased under unfavourable culture conditions or longer passages.

Upon infection with A-MuLV, the two cell types developed completely opposite growth phenotypes; although both underwent morphological change characteristic of A-MuLV transformed cells (Figure 5C). The analyses shown in Figure 5 were performed on whole populations of infected cells at 3−5 days post-infection. The infected 1A4− cells became growth arrested and they did not proliferate even in medium containing serum (Figure 5B). Infected 4A2+ cells did not become growth arrested, but instead acquired the ability to proliferate in serum-free medium (Figure 5B). Immunoblotting with the anti-Abl or the anti-Ptyr antibodies showed no difference in the expression of the v-Abl tyrosine kinase in these polyclonal populations of infected cells (Figure 5A). These results demonstrated that the development of the GS or the GI phenotype was determined by the cell context, but not by mutation or the levels of expression of the v-*abl* oncogene. Only a subset of NIH3T3 cells, exampled by the 4A2+ clone, could be transformed by A-MuLV. A recent report has indicated that v-Abl can interact with signal transducers, such as PI 3-kinase (Varticovski *et al.*, 1991). We could reproduce that result by showing that a phosphatidylinositol (PI) kinase activity could be immunoprecipitated with antibodies for

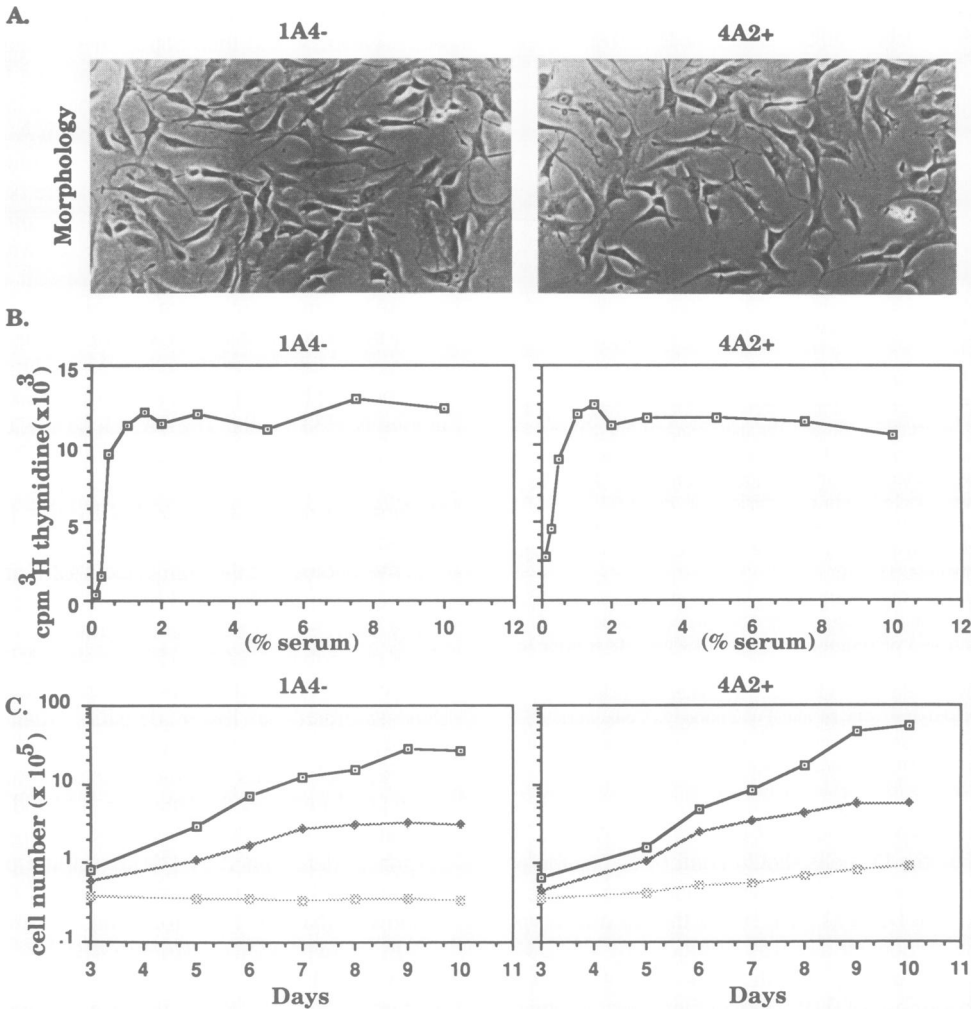

Fig. 4. Morphology and growth characteristics of the positive and negative NIH3T3 subclones. **(A)** Photographs of phase contrast microscopy of uninfected 1A4− and 4A2+ cells growing in 10% serum (magnification of ×25). **(B)** Growth response in different concentrations of serum. Quiescent 1A4− and 4A2+ cells were stimulated with the indicated concentrations of serum. 14 h after the addition of serum, [^{3}H]thymidine was added (1 μCi/ml) and the incorporation measured after a 2 h pulse. Stimulation reached a plateau at 1% serum for both clones. **(C)** Growth curves in different concentrations of serum. 1A4− and 4A2+ cells were each seeded in 18 6 cm plates (1×10^4 cells per plate) in 10% serum to allow for optimal attachment. After 12 h (day 0), the medium was replaced with either 10% (open squares), 1% (filled diamonds) or 0.33% (filled squares) serum. Cells were fed fresh medium daily, and live cells were counted by trypan blue exclusion, on the indicated days. Growth rates on days 1 and 2 were similar for all serum concentrations due to the 12 h pre-incubation in 10% serum. Growth rates from the third day on were representative of the indicated concentration of serum.

phosphotyrosine (anti-Ptyr) or for v-Abl (anti-Abl) from A-MuLV infected cells (Figure 5D). In mock-infected cells, no PI kinase activity was detected in anti-Ptyr (Figure 5D) or anti-Abl immunoprecipitates (not shown). The PI kinase activity was precipitated by the two antibodies from A-MuLV infected 1A4 or 4A2 cells (Figure 5D). Thus, the association of v-Abl with PI kinase, like shape change, is not correlated with the growth phenotypes.

The positive or negative cell context also affected the transactivating function of v-Abl tyrosine kinase. This could be demonstrated in transient co-transfections of a plasmid containing the A-MuLV genome with a reporter plasmid FC1, which contained the c-*fos* promoter (−2250 to +1) linked to the chloramphenicol acetyltransferase gene (CAT) (Deschamps *et al.*, 1985). When co-transfected into the 4A2+ cells, an average of 7.4-fold induction of CAT expression by the v-Abl tyrosine kinase was observed (Figure 6). The induction was dependent on the tyrosine kinase activity because a kinase-defective v-*abl* mutant failed to activate the c-*fos* promoter (Figure 6). In the 1A4− cells, however, v-Abl did not activate the c-*fos* promoter (Figure 6). The c-*fos* promoter was activated by serum in both cell types, showing that the normal mitogenic pathway is operative in the 1A4− cells. By infecting the 4A2+ and the 1A4− cells with temperature sensitive A-MuLV, it could be shown that the endogenous c-*fos* and c-*myc* were induced, by temperature shift, in the 4A2+ cells only. These results demonstrate that the 4A2+ cells express a function that allows the v-Abl tyrosine kinase to activate serum-inducible genes. Without this function, as is with the 1A4− cells, the v-Abl tyrosine kinase has no mitogenic activity.

Conversion of the negative NIH3T3 cells by 5-azacytidine

A large number ($>10^6$) of A-MuLV infected 1A4− cells have been plated in serum-free medium or in soft agar without giving rise to any detectable growth. Thus, the rate of spontaneous conversion to the positive phenotype was

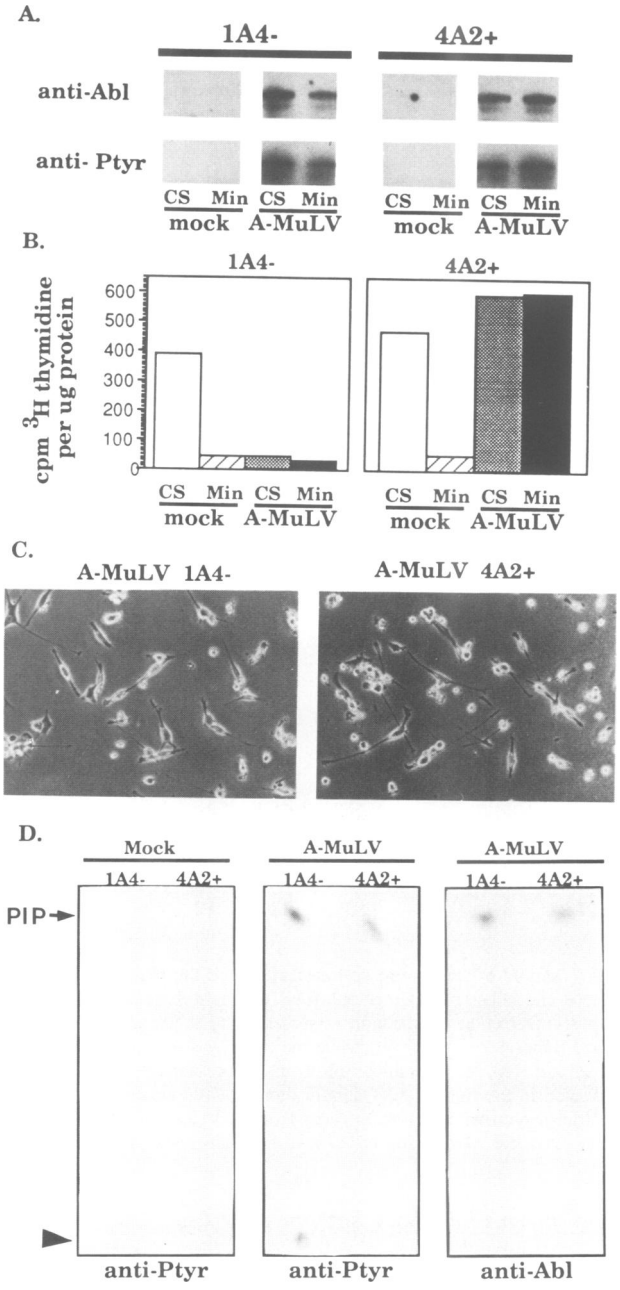

Fig. 5. Differential growth response induced by the v-Abl tyrosine kinase in the two NIH3T3 cell types. 1A4− and 4A2+ cells were infected with either A-MuLV, or Moloney virus (mock) at an m.o.i. of 5. After 3 days, whole populations of infected cells were fed either 10% serum (CS) or serum-free medium (Min) and incubated for an additional 48 h. The growth phenotypes were determined on day 5 post-infection. **(A)** Immunoblotting of infected cell lysates with anti-Abl or anti-Ptyr antibodies. Autoradiographs of western blots containing 50 μg of each lysate were exposed for 9 h at room temperature. Endogenous c-Abl could be detected in mock infected lysates with longer exposures. **(B)** Cell proliferation was measured by the incorporation of [^{3}H]thymidine after a 2 h pulse labelling, in 10% serum (CS) or serum-free medium (Min) as indicated. **(C)** Morphology of A-MuLV infected 1A4− and 4A2+ in 10% serum. Photographs were taken 3 days after infection on whole populations of infected cells using phase contrast microscopy (magnification ×25). **(D)** PI kinase activity in immunoprecipitates. Mock- or A-MuLV-infected 1A4− or 4A2+ cells were lysed and incubated with polyclonal antibodies for phosphotyrosine (anti-Ptyr) or for the C-terminal region of the Abl protein (anti-Abl, CTN2). The immunoprecipitates were washed and used in PI kinase assays as described in Materials and methods. The position of the TLC origin is indicated by the arrow head.

Table II. Conversion of negative NIH3T3 cells by 5-azacytidine

Cell	[5-aza-CR][a] (μM)	Number of colonies in soft agar[c]	
		mock-infection[b]	A-MuLV infection[b]
1A4−	0	0	0
1A4−	1	0	30
1A4−	3	0	243
4A2+	0	0	4710
4A2+	1	0	4160
4A2+	3	0	4475

[a]1A4− and 4A2+ cells were each plated in six seperate plates and then treated in duplicate with 5-azacytidine at the indicated concentrations.
[b]After 24 h the 5-azacytidine was removed and the cells washed once with medium, one set of the duplicate plates was then infected with A-MuLV while the other was mock-infected.
[c]At 3 days post-infection, cells were plated in soft agar (without 5-azacytidine) and the number of soft agar colonies was counted after 2 weeks (post-infection). Only colonies larger than 0.5 mM (>500 cells) were scored.

$<10^{-6}$. The stability of the two phenotypes suggested a genetic difference between the two NIH3T3 cell types. The 5−8% frequency of the positive cells, on the other hand, was too high to be accounted for by the rate of mutation. A possible cause for the high frequency and the stability of the positive trait could be a change in DNA methylation. Undermethylation of DNA has been seen in a large number of animal and human tumour cells, implicating DNA hypomethylation in tumour development (Jones, 1986). Therefore we tested whether the phenotypes of these two NIH3T3 cell types could be affected by 5-azacytidine, which inhibits DNA methylation (Jones and Taylor, 1980). Interestingly, treatment of the 1A4− cells with 5-azacytidine was found to convert them to the positive phenotype, albeit at a low frequency (Table II). 5-azacytidine alone did not induce the formation of soft agar colonies in either the 1A4− or the 4A2+ cells (Table II). However, when 5-azacytidine treated 1A4− cells were infected with A-MuLV, soft agar colonies were formed and the number of colonies increased with the concentration of 5-azacytidine (Table II). Infected 4A2+ cells formed a large number of soft agar colonies and their numbers were not affected by 5-azacytidine (Table II). These results indicated that the positive cell trait could be induced by the demethylation of the DNA. Because hypomethylation of DNA is usually correlated with the activation of gene expression (Razin and Riggs, 1980; Hsiao *et al.*, 1984; Jones, 1985; Razin and Cedar, 1991), the difference between the two cell types may be due to the expression of one or more cellular genes.

Discussion

The oncogenic v-Abl tyrosine kinase can inhibit or stimulate proliferation depending the context of an NIH3T3 cell. We have isolated two types of NIH3T3 cells that can be distinguished by their response to v-Abl. In the 'positive' NIH3T3 cells, represented by clone 4A2+, v-Abl tyrosine kinase activates the mitogenic pathway. In the 'negative' NIH3T3 cells, represented by clone 1A4−, v-Abl tyrosine kinase does not activate mitogenesis, but instead causes a G_1 arrest. In both types of NIH3T3 cells, v-Abl tyrosine kinase induces shape change typical of transformed

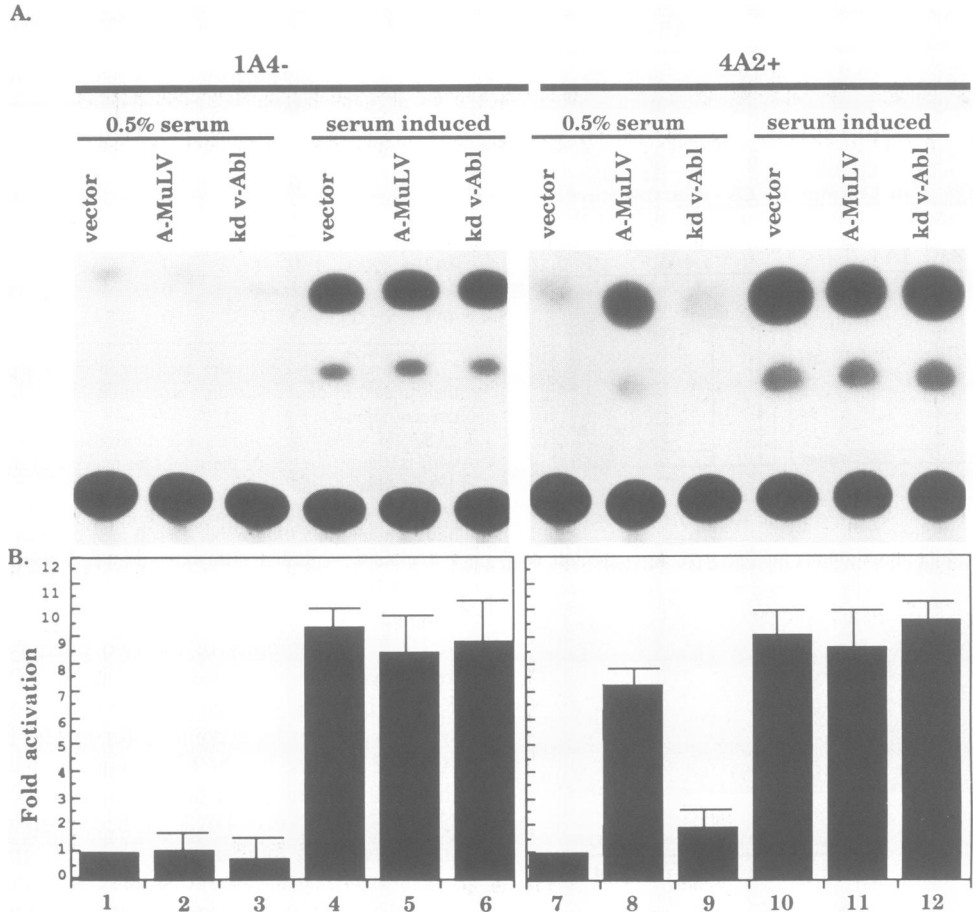

Fig. 6. V-*abl* activates the c-*fos* promoter only in the positive NIH3T3 cells. 1A4− and 4A2$^+$ cells were co-transfected with the plasmid FC1, which contained −2250 to +1 of the human c-*fos* promoter linked to the CAT gene and either pSP65 (vector), pAB160 (A-MuLV) or a plasmid expressing a kinase defective mutant of A-MuLV (kd v-Abl), in duplicate. 24 h post-transfection the medium was changed to 0.5% serum and cells were incubated for an additional 48 h. One set of the duplicates was then harvested (lanes 1−3 and 7−9), while the other was induced by the addition of 20% serum (lanes 4−6 and 10−12) 4 h prior to harvesting for CAT assays. **(A)** An autoradiograph from a representative CAT assay. Assays were incubated for 1 h using 75 μg of cell extract. The cell types are indicated in the figure. **(B)** Quantification of CAT assay results. The CAT activities were measured and compared with the uninduced level, i.e. in co-transfection with the vector (lane 1 or 7). Values were adjusted to the amount of CAT DNA found in each transfection (Materials and methods), to account for transfection efficiency. The values shown are the average of three separate experiments.

fibroblasts. Thus, morphological alteration is not necessarily an indicator of neoplastic transformation by v-Abl. The dissociation of morphological transformation from factor-independent growth has also been reported by others studying the v-*abl* oncogene (Rees-Jones *et al.*, 1989).

The positive context can be introduced by cell fusion, for instance, fusion of a temperature sensitive-A-MuLV infected 1A4− cells with uninfected 4A2+ cells causes serum- and anchorage-independent growth at 32°C, but not at 39°C (M.W.Renshaw, unpublished observations). The positive cell context can also be induced in the negative cells by treatment with 5-azacytidine. Transfection of the negative cells with the genomic DNA of the positive cells, followed by infection with A-MuLV, caused the formation of 1−2 soft agar colonies in a preliminary experiment (M.W.Renshaw, unpublished observations); indicating that the positive cell context might also be transferred by DNA. This is reminiscent of the experiment in which transfection of untreated 10T1/2 cells with genomic DNA of the 5-azacytidine-induced myoblasts caused a myoblastic conversion (Lassar *et al.*, 1986). These observations suggest that the difference in the cell context may originate from the differential expression of normal cellular genes.

The growth inhibitory activity of V-Abl

The primary biological effect of v-Abl in a majority of NIH3T3 cells is to cause a G_1 arrest. This activity could account for the previously reported 'lethal' effect of A-MuLV (Ziegler *et al.*, 1981; Goff *et al.*, 1982; Watanabe and Witte, 1983). We found that in D4X cells, the induction of c-*fos* mRNA by serum was severely reduced at 32°C, but not at 39°C. The serum-induced c-*myc* expression, however, was unaffected by v-Abl (E.T.Kipreos, unpublished). The inhibition of serum-induced expression of c-*fos* by v-Abl was not observed in transient co-transfection assays (Figure 6), suggesting that the inhibition might require a prolonged exposure to the v-Abl activity. The reduction in c-*fos* mRNA alone could not account for the growth arrest, because the constitutive expression of v-*fos* in D4X cells did not reverse the G_1-arrested phenotype (E.T.Kipreos, unpublished). It is possible that v-Abl might reduce the expression of genes other than c-*fos* to cause the cell cycle arrest.

The selective inhibition of c-*fos* induction by v-Abl suggests that the growth arrest may result from a specific block in the mitogenic pathway. We have found that v-Abl interacts with PI kinase in 1A4 cells that are growth-arrested.

It is conceivable that the interaction between v-Abl and signal transducers such as PI kinase may contribute to the G_1 arrest. For instance, v-Abl might sequester the transducers from the receptor tyrosine kinases to block the mitogenic pathway. Alternatively, the constitutive interaction with signal transducers may induce a feedback control to inhibit proliferation.

The decreased cell attachment induced by v-Abl might also contribute to the growth arrest. Fibroblastic cells require attachment to grow and detachment is shown to cause G_1 arrest (MacPherson and Montagnier, 1964; Otsuka and Moskowitz, 1976; Campisi and Medrano, 1983). Although plating with fibronectin did not rescue the A-MuLV infected cells, co-cultivation with normal NIH3T3 cells did allow them to form small foci. The supporting monolayer might provide critical factors through cell−cell interaction or junctional communication to reverse the inhibitory effect of v-Abl.

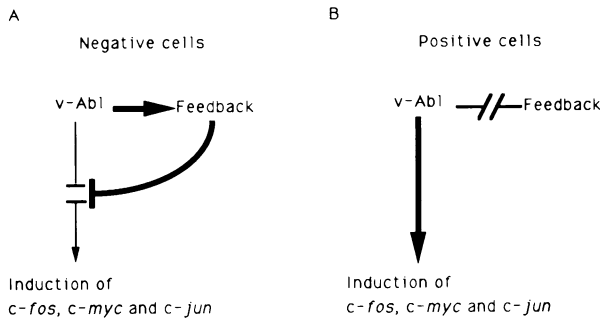

Fig. 7. A model for the growth inhibitory activity of v-Abl and the nature of the permissive cell context. **(A)** Constitutive expression of the v-Abl tyrosine kinase results in the induction of a feedback control mechanism, which blocks the mitogenic activity of v-Abl and of the normal receptor tyrosine kinases to cause growth arrest in the negative NIH3T3 cells. **(B)** In positive NIH3T3 cells the feedback control pathway is non-functional, thus in these cells v-Abl can induce mitogenesis and transformation.

The permissive cell context

Amplification of the c-*myc* gene and an accumulation of the p53 protein have been found in A-MuLV transformed lymphoid cells (Rotter *et al.*, 1980; Nepveu *et al.*, 1985). We therefore examined the positive and the negative NIH3T3 cells for the expression of those two genes. Comparable levels of c-*myc* mRNA were found in both cell types. The two cell clones also produced similar levels of p53 protein that had a half-life of 1−2 h, indicating that both lines might express mutated p53 (M.W.Renshaw, unpublished). Thus, c-*myc* and p53 are not directly responsible for the permissive context.

Although the 4A2 and the 1A4 clones were isolated by their response to v-Abl, we found that they also showed a differential response to the v-Src tyrosine kinase. Transfection with v-*src* induced foci only in the 4A2+ cells but not in the 1A4− cells (M.W.Renshaw, unpublished). The Src-foci of 4A2 cells were fully transformed as they grew in soft agar and in serum-free medium. Thus, morphological alteration is an indicator of transformation by the v-*src* oncogene. Transformation by the H-*ras* and K-*ras* oncogenes, however, was not dependent on the positive cell context because both 4A2+ and 1A4− cells were transformed by the two *ras* oncogenes. These observations suggest that the positive cell context may act downstream from the oncogenic tyrosine kinases but upstream of v-Ras in the mitogenic pathway.

The mitogenic activity of receptor-activated tyrosine kinases, unlike v-Abl and v-Src, is not affected by the permissive context; because both types of NIH3T3 cells show a normal response to serum or to purified platelet-derived growth factor. Both cell types are dependent on growth factors and they proliferate at similar rates, however, the 4A2 cells can grow to higher densities than the 1A4 cells at several different serum concentrations. This suggests that a part of the mitogenic pathway may have become deregulated in the 4A2 cells.

It is possible that tyrosine kinases like v-Abl and v-Src do not activate all the signal transducers that are required to stimulate cell growth. In the positive cells, a collaborating function may be expressed to allow v-Abl and v-Src to stimulate growth. The collaborating function could be in the form of a connector linking v-Abl to the mitogenic pathway. On the other hand, some signal transducers may become constitutively activated in the positive cells to collaborate with the v-Abl tyrosine kinase. This model, however, does not explain the G_1 arrest induced by v-Abl.

We favour an alternative model, depicted in Figure 7, which explains both the growth inhibitory activity of v-Abl and the nature of the permissive cell context. We propose that by itself v-Abl tyrosine kinase is a competent activator of the mitogenic program. However, the constitutive expression of an activated tyrosine kinase such as v-Abl also induces a feedback control, a built-in safety net, which blocks the mitogenic activity of v-Abl and of the normal receptor tyrosine kinases to cause G_1 arrest. In the positive NIH3T3 cells, the feedback control is inactivated. Loss of the feedback control strips the cells of the defence against deregulated tyrosine kinase, such as v-Abl and v-Src, and allows the induction of neoplastic transformation. Since the positive context is dominant and inducible with 5-azacytidine, the feedback control could be lost through the overproduction of a dominant negative mutant protein or through the expression of a dominant positive inhibitor that severs the feedback pathway. This model could explain the growth inhibitory activity of the v-Src tyrosine kinase in 3T3-TNR9 cells (Nori *et al.*, 1990) and of the epidermal growth factor (EGF) in A431 cells, where high concentrations of EGF or high levels of the receptor cause an EGF-induced inhibition of cell proliferation (Lifshitz *et al.*, 1983; Kawamoto *et al.*, 1984). Exploration of the genetic difference between the 4A2+ and the 1A4− cells may shed light on the regulation of the mitogenic pathway.

Implication on the disease specificity of A-MuLV

Our finding that transformation by v-*abl* requires a dominant cell context is consistent with the proposal that immortalized rodent cells express collaborating functions to allow transformation by a single oncogene (Weinberg, 1989). Immortalization may be sufficient to collaborate with the activated *ras* oncogenes, but our results show that immortalization alone is not sufficient to make cells sensitive to transformation by all oncogenes. Although v-*abl* does not transform primary fibroblasts, it is a potent transforming agent of pre-B lymphocytes both *in vivo* and *in vitro* (Rosenberg *et al.*, 1975). Perhaps pre-B cells are naturally endowed with the permissive context to allow the manifestation of the mitogenic activity of v-Abl, hence, their susceptibility to transformation by A-MuLV.

Materials and methods

Cell culture

NIH3T3 (Lewis clone 7) cells were cultured in Dulbecco's modified Eagle's medium with 10% defined/supplemented bovine calf serum (Hyclone). Soft agar colonies were selected in 0.6% Bacto-agar. Growth factor-independent colonies were selected in serum-free medium that contained 3:1 DMEM:Hams F12 supplemented with 0.075% BSA (Fraction V), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Sigma). Mitomycin C treatment (25 μg/ml) of NIH3T3 cells was performed on trypsinized cells for 30 min, cells were then washed three times with growth medium and used in co-cultivation experiments. 5-azacytidine treatment has been previously described (Taylor and Jones, 1979). Briefly, fresh stocks of 5-azacytidine were mixed in phosphate buffered saline (PBS), filter sterilized and then added to the plates at final concentrations of 0.3, 1.0 or 3.0 μM. After incubation for 24 h, 5-azacytidine was removed, the cells washed twice with medium and then infected with A-MuLV. No 5-azacytidine was present in the medium during the infection or subsequent plating in soft agar.

Clonal isolation of cell lines

Growth inhibited clones such as D4X were isolated by infecting NIH3T3 cells with A-MuLV DP160 virus (Kipreos and Wang, 1988) at an m.o.i. of 10, followed by limiting dilution into 96 well plates at 39°C. Clones were checked for the expression of v-Abl protein by immunoblotting with the anti-Abl antibody 8E-9. The growth-stimulated clone D5 was isolated as a soft agar colony at 32°C after infection with the same virus. Subclones of NIH3T3 cells were isolated by limiting dilution in 96 well plates. Ninety-four separate clones were obtained and 10^3 cells from each clone were infected with A-MuLV and plated in soft agar. The clone 4A2 was one of eight that formed numerous large soft agar colonies, while the clone 1A4 was one of 57 that formed no soft agar colonies upon A-MuLV infection. The other 29 clones formed intermediate numbers of soft agar colonies and might have been derived from more than one cell.

Growth rate and cell cycle analysis

Cell proliferation was measured by the incorporation of [^{3}H]thymidine after a 2 h pulse labelling with 1 μCi/ml, following the procedure previously described (Morla *et al.*, 1989). The cell cycle distribution was determined by flow cytometry analysis of propidium iodide stained cells (Morla *et al.*, 1989).

Statistical analyses and calculations

To determine the frequency of the growth stimulatory phenotype among A-MuLV infected cells, NIH3T3 cells were infected at an m.o.i. of 0.01 and seeded at different densities (125–8000 cells/well) in 96 well plates. Each density was seeded 192 times (two plates). One set of plates was fed medium containing 10% serum, while the other was cultured in serum-free medium. Foci were scored after 15 days. Calculations, using the binomial distribution $(p + q)^n = 1$, were based on the number of wells that contained no foci, because the fate of each cell in those wells was known. For example, when 125 cells/well were plated in regular medium, 24 out of 96 wells contained no foci. From this, the per cell probability of not forming a focus, q, was determined ($q^{125} = 24/96$, thus $q = 0.98897$). Having determined q, the per cell probability of forming a focus, p, was calculated to be 0.01103, because $p = 1 - q$. This number was virtually identical to the m.o.i. (0.01), indicating that every A-MuLV infected cell formed a focus in regular medium. The per cell probability of forming a focus in serum-free medium was calculated to be 0.00055. Thus, the frequency at which an A-MuLV infected cell became serum-independent was 4.99% (0.00055/0.01103). The average frequency, calculated from seven different densities of plating and two independent experiments, was 5% ± 1%.

To determine the theoretical frequency of infection as a function of m.o.i., calculations were made using the Poisson distribution $p(K) = e^{-m}m^k/k!$, where m is the m.o.i. and k is the number of virus in an infected cell. m.o.i. is defined as focus-forming units of A-MuLV/cell number. Calculations were based on a hypothetical mutant virus comprising 5% of the total viral population. Thus, when the total viral m.o.i. was 20, the mutant virus m.o.i. would be 1 (5% of 20 = 1) and the frequency of infection by the mutant virus is $1 - e^{-1} = 0.63$, i.e. 63% of the cells should be infected by at least one mutant virus.

Induction of serum-responsive genes

RNA was extracted by the guanidinium thiocynate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Northern blots (Maniatis *et al.*, 1982) contained 20 μg total RNA per lane and were hybridized to random primer labelled probes (Feinberg and Vogelstein, 1983). The murine probes used were: a β_2 microglobulin 240 bp *Eco*RI–*Pst*I fragment from pSPT672 (Parnes and Seidman, 1982), a c-*fos* 1.1 kb *Ava*I fragment from pFBJ-2 (Van Beveren *et al.*, 1983), a c-*myc* 580 bp *Pst*I fragment from pM c-myc 54 (Stanton *et al.*, 1983) and a c-*jun* 800 bp PstI–*Hin*dIII fragment of from pRSVc-Jun (Chiu *et al.*, 1988). Hybridization and washing were performed under standard conditions and autoradiographs were scanned with a laser densitometer. Protein synthesis was inhibited with cyclohexamide 25 μg/ml (Müller *et al.*, 1984). Nuclear run-off assays were performed as described by Linial *et al.* (1985) measuring the transcription rates of c-*fos*, c-*myc* and β_2 microglobulin in nuclear extracts isolated from D5 cells shifted to 32°C for 1.5 h. Downregulation of protein kinase C with TPA and inhibition with H7 were performed as previously described (Collins and Rozengurt, 1984; Hidaka *et al.*, 1984).

Transient co-transfection assays

Cells were seeded at 5×10^5 cell/10 cm dish and were transfected by the calcium phosphate procedure (Graham and Van Der Eb, 1973) after 12 h with 2 μg of the plasmid FC1 (Deschamps *et al.*, 1985) and 10 μg of either pSP65 (Pharmacia), pAB160 (Latt *et al.*, 1983), which contains the A-MuLV coding sequence, or pAB160His– (where Lys295 at the ATP binding site is substituted with His, J.Y.J.Wang, unpublished) expressing a kinase defective mutant of v-*abl*. CAT assays were performed as previously described (Gorman *et al.*, 1982), using equal amounts of protein extracts prepared from half of the cells. DNA was isolated from the other half for southern blot analysis, probing with a 250 bp *Hin*dIII–*Eco*RI fragment of the CAT coding sequence. The CAT assay results were normalized with the CAT DNA.

Assay of PI kinase activity

A-MuLV and Mock-infected 1A4– and 4A2+ cells (5×10^6 per 15 cm plate) were incubated in 0.5% CS for 48 h and then lysed in 2 ml of 1% NP40, 20 mM Tris–HCl pH 7.8, 50 mM NaCl, 50 mM NaF, 5 mM EDTA and 1 mM sodium orthovanadate. Lysates were precleared by ultracentrifugation at 100 000 *g* for 30 min at 4°C and then incubated for 6 h with purified antibodies for either phosphotyrosine (anti-Ptyr) or the C-terminal region of the Abl protein (anti-Abl, CTN-2). Immune complexes were collected on protein A–Sepharose beads. The beads were extensively washed and assays for PI Kinase activity were performed as previously described (Varticovski *et al.*, 1991).

Cell fusion experiments

Antibiotic resistant clones of D5, D4X and NIH3T3 cells were established by transfection with plasmids expressing hygromycin or neomycin resistance genes. Stable clones expressing the selected antibiotic resistance were plated in soft agar and in serum free medium at 39°C and 32°C to verify that they maintained the parental phenotype. For fusions, 5×10^5 cells from each line were plated in a 6 cm dish. After 24 h, cells were fused with 52.5% PEG 1000 according to the procedures previously described (Davidson and Gerald, 1976; Davidson *et al.*, 1976). Hybrids were selected by double antibiotic resistance at the restrictive temperature, 39°C, where v-Abl was inactive and would not bias the selection for a given growth phenotype. To confirm that the hybrids were derived from the fusion of two cells, the number of chromosomes was counted by swelling cells in 0.8% sodium citrate for 20 min, fixing in methanol–acetic acid (3:1), and then mounting on to a slide for visualization of the mitotic chromosomes. The v-Abl-induced phenotypes of hybrid clones were tested by plating cells in soft agar or in medium with and without serum at 32°C and 39°C. The ability of v-Abl to induce c-Fos protein was tested at 32°C, using indirect immunofluorescence with an anti-Fos antibody as detailed by De Togni *et al.* (1988).

Immunoblotting

Cells were lysed in SDS–PAGE sample buffer (67 mM Tris–HCl pH 6.8, 10 mM EDTA, 2% SDS, 10% glycerol, 0.3% 2-mercaptoethanol and 0.03% bromophenol blue) by boiling for 15 min. Protein was quantified by the method of Lowry *et al.* (1951), 50 μg samples were then run on 7.5% SDS–PAGE gels and transferred to Immobalon. Immunoblotting with anti-Abl and anti-Ptyr antibodies was performed as previously described (Wang, 1985; Richardson *et al.*, 1987). Quantification of v-Abl protein and Ptyr levels was achieved by laser densitometric scanning of autoradiographs from western blots.

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