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# The cytostatic function of c-Abl is controlled by multiple nuclear localization signals and requires the *p53* and *Rb* tumor suppressor gene products

**Shih-Te Wen, Peter K.Jackson[1] and Richard A.Van Etten[2]**

Center for Blood Research, Department of Genetics and [1]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA

[2]Corresponding author

**c-Abl is a non-receptor protein-tyrosine kinase lacking a clear physiological role. A clue to its normal function is suggested by overexpression of Abl in fibroblasts, which leads to inhibition of cell growth. This effect requires tyrosine kinase activity and the Abl C-terminus. c-Abl is localized to the cell nucleus, where it can bind DNA, and interacts with the retinoblastoma protein, a potential mediator of the growth-inhibitory effect. Nuclear localization of Abl can be directed by a pentalysine nuclear localization signal in the Abl C-terminus. Here, we have identified two additional basic motifs in the Abl C-terminus, either of which can function independently of the pentalysine signal to localize Abl to the nucleus. Using a quantitative transfection assay, we show that both c-Abl and transforming Abl proteins inhibit entry into S phase and this effect is absolutely dependent on nuclear localization. Further, we demonstrate that the Abl cytostatic effect requires both the *Rb* and *p53* tumor suppressor gene products. These results indicate that Abl inhibits cell proliferation by interacting with central elements of the cell cycle control apparatus in the nucleus, and suggest a direct connection between p53 and Rb in this growth-inhibitory pathway.**

*Keywords*: Abelson murine leukemia virus/p53/retinoblastoma protein/tyrosine kinase

## Introduction

The c-*abl* proto-oncogene, first identified as the normal cellular homolog of the transforming gene of Abelson murine leukemia virus (Ab-MuLV), encodes a non-receptor protein-tyrosine kinase related to the Src family. The N-terminal region of Abl is structurally similar to Src and includes Src homology regions 3 and 2 (SH3 and SH2) (Pawson and Schlessinger, 1993) and the catalytic domain; however, Abl differs from all Src family members by the presence of a large (~90 kDa) C-terminal domain. Several functional motifs have been defined in the Abl C-terminus, including a nuclear localization signal (NLS) (Van Etten *et al.*, 1989), DNA binding domain (Kipreos and Wang, 1992), actin binding domain (McWhirter and Wang, 1993; Van Etten *et al.*, 1994), sites for phosphorylation by protein kinase C (Pendergast *et al.*, 1987) and cdc-2 kinase (Kipreos and Wang, 1990), and binding sites for the adapter proteins Crk, Grb-2 and Nck (Ren *et al.*, 1994).

The physiology of Abl has been best elucidated by the study of its transforming variants. Normal c-Abl is unable to transform cells, even if overexpressed (Jackson and Baltimore, 1989), but can be activated to transform fibroblasts and hematopoietic cells by several distinct mechanisms, including deletions (Franz *et al.*, 1989; Jackson and Baltimore, 1989) or point mutations (Van Etten *et al.*, 1995) in the SH3 domain, substitution of polypeptides derived from retroviral *gag* (Van Etten *et al.*, 1995), *BCR* (Shtivelman *et al.*, 1985; Stam *et al.*, 1985) or *TEL* (Papadopoulos *et al.*, 1995) genes at the extreme N-terminus, and point mutations in the kinase domain (Jackson *et al.*, 1993b). Fibroblast transformation by activated Abl requires tyrosine kinase activity (Rosenberg *et al.*, 1980; Engelman and Rosenberg, 1987; Kipreos *et al.*, 1987), the phosphotyrosine binding function of the SH2 domain (Mayer *et al.*, 1992) and myristoylation (Daley *et al.*, 1992). The functions of the c-*ras* (Smith *et al.*, 1986; Stacey *et al.*, 1991; Sawyers *et al.*, 1995) and c-*myc* (Sawyers *et al.*, 1992) genes are required for Abl transformation, and activated Abl has been identified recently as a potent stimulator of the stress-activated protein kinase pathway (Sanchez *et al.*, 1994).

In contrast, little is known about the physiological role of the normal c-Abl protein. In the mouse, c-*abl* mRNA is widely expressed throughout embryonic and adult life, with highest levels in thymus and spleen (Muller *et al.*, 1982; Ben-Neriah *et al.*, 1986; Renshaw *et al.*, 1988). Mice with homozygous inactivation of the c-*abl* locus display a strong tendency for runted growth and neonatal death, and exhibit thymic and splenic atrophy with deficiencies of B and T lymphoid progenitors (Schwartzberg *et al.*, 1991; Tybulewicz *et al.*, 1991), supporting a role for Abl in lymphoid development.

Other important clues to the physiological role of c-Abl have come from studies of the subcellular localization of the protein. The myristoylated form (type Ib/IV) of c-Abl is largely nuclear in location in fibroblasts (Van Etten *et al.*, 1989) and hematopoietic cells (Wetzler *et al.*, 1993). Interestingly, activated forms of Abl are localized exclusively to the cytoplasm and plasma membrane of transformed cells when analyzed by immunofluorescence (Van Etten *et al.*, 1989; Daley *et al.*, 1992; Wetzler *et al.*, 1993). Deletion and site-specific mutagenesis defined a pentalysine (K5) motif in the C-terminus as the major NLS of Abl; however, other evidence suggests that an additional NLS is present elsewhere in the C-terminus of Abl (Van Etten *et al.*, 1989; McWhirter and Wang, 1991).

The function of nuclear Abl is not understood. The C-terminus of Abl contains a DNA binding domain (Kipreos and Wang, 1992) which may confer sequence-specific DNA binding (Dikstein *et al.*, 1992); phosphorylation of the Abl C-terminus by cdc-2 kinase during mitosis blocks DNA binding (Kipreos and Wang, 1992). A fraction of nuclear

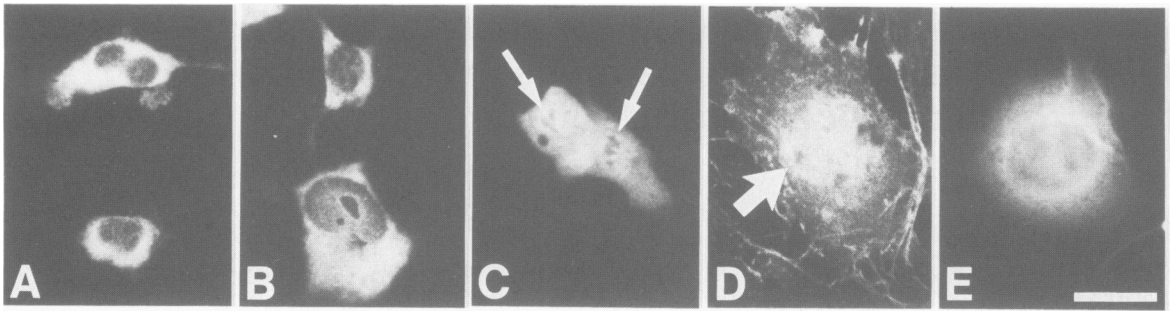


**Fig. 1.** Identification of additional nuclear localization signal(s) in the c-Abl C-terminus. (**A–C**) Localization of pyruvate kinase (PK)–Abl fusion proteins after transient expression in 293T cells. The indicated fusion protein was localized by indirect immunofluorescence utilizing anti-PK antibodies. (A) PK (parental); (B) PK–SH3; (C) PK–Abl C-terminus. (**D** and **E**) Localization of GST–Abl fusion protein after microinjection into NIH 3T3 cells, utilizing anti-GST antibodies. (D) GST–pEX4; (E) GST (parental). Bar = 10 μm.

c-Abl binds directly with the Rb-1 tumor suppressor protein in a cell cycle-dependent manner (Welch and Wang, 1993). The C-terminus of Rb interacts with the ATP binding region of the c-Abl kinase domain, inhibiting Abl kinase activity in G1, while hyperphosphorylation of Rb results in release of Abl and activation of Abl kinase activity in S phase. Abl has no intrinsic transcriptional activating function (S.-T.Wen and R.A.Van Etten, unpublished data), but appears to enhance transactivation by other transcription factors in a manner which requires kinase activity and DNA binding and is inhibitable by Rb (Welch and Wang, 1993). Transcriptional activation by Abl might be mediated by tyrosine phosphorylation of the C-terminal domain of RNA polymerase II (Baskaran *et al.*, 1993). Taken together, these results suggest that nuclear c-Abl may play a positive role in cell growth during S phase.

One striking phenotype associated with Abl is its strong growth-suppressive effect, observed both with transforming Abl (Ziegler *et al.*, 1981; Goff *et al.*, 1982; Watanabe and Witte, 1983) and c-Abl. NIH 3T3 clones overexpressing c-Abl 10- to 20-fold over endogenous Abl exhibit slowed growth with prolongation of the $G_1$ phase of the cell cycle, and such clones are very unstable, with loss of Abl expression with continued passage of the cells (Van Etten *et al.*, 1989). Further, a marked reduction in the recovery of drug-resistant colonies is observed upon co-transfection of fibroblasts with c-*abl* and a selectable marker; elimination of tyrosine kinase activity or deletion of the Abl C-terminus improves protein expression and abolishes the reduction in recovery of a selectable marker after co-transfection (P.Jackson and R.A.Van Etten, unpublished data). Conditional overexpression of c-Abl causes growth arrest in $G_1$, and deletion of the SH2 domain or the pentalysine NLS improves the stability of expression of the protein after transfection (Sawyers *et al.*, 1994). These results demonstrate that a growth-inhibitory effect is observed upon expression of transforming Abl or c-Abl in fibroblasts, and suggest that both kinase activity and the Abl C-terminus are required for the effect.

The requirement for the C-terminus for growth inhibition by Abl suggests that this effect is mediated by one or more distinct functional motifs within this large region, and explains why similar toxicity is not observed with any of the Src family members, which lack this domain. The mapping of an NLS to the Abl C-terminus (Van Etten *et al.*, 1989) suggested that nuclear localization might be required for the negative growth effect of Abl (Sawyers *et al.*, 1994). However, assessment of the requirement for nuclear localization is complicated by the presence of additional C-terminal signals capable of mediating nuclear localization of Abl (Van Etten *et al.*, 1989; McWhirter and Wang, 1991) and the inadequacy of transient transfection assays in heterologous cells such as COS cells to predict localization of Abl in fibroblasts (Van Etten *et al.*, 1989; Goga *et al.*, 1993). In addition, it has been difficult to understand the paradox that transforming Abl proteins, while wholly cytoplasmic by immunofluorescence analysis, are nevertheless still toxic. In this study, we have defined two additional conserved sequences in the Abl C-terminus which can function independently in a manner necessary and sufficient to localize Abl to the nucleus. In a quantitative transfection assay, we show that both native and transforming c-Abl proteins induce a retardation or block in $G_1$, and that all three NLS contribute to this effect. We also show that the cytostatic effect of Abl requires both the *p53* and *Rb* tumor suppressor gene products. These results demonstrate that Abl acts in the nucleus to inhibit cell growth through central elements of the cell cycle machinery, and imply that p53 and Rb are linked directly in this cytostatic pathway.

## Results

### *Additional nuclear localization signal(s) in the Abl C-terminus*

C-terminal truncation mutants of murine type IV c-Abl established that a pentalysine (K5) NLS was sufficient to confer nuclear localization of Abl in NIH 3T3 cells, and a site-specific mutant of Abl with the pentalysine motif altered to glutamines was localized cytoplasmically after transient expression in COS cells (Van Etten *et al.*, 1989). However, when the same mutant, c4 $1NLS^Q$, was stably expressed in NIH 3T3 cells, the protein exhibited a subcellular localization similar to that of wild-type c-Abl, largely nuclear (Van Etten *et al.*, 1989) (Figures 2A and 3A). Because the mutant protein c4 ΔBcl (Van Etten *et al.*, 1989), which is truncated just before the K5 NLS, was cytoplasmic in NIH 3T3 cells (data not shown), this implied the existence of an additional NLS C-terminal to the K5 signal, and suggested that the functioning of the different Abl NLS was dependent on the cell type.

To confirm the existence of additional NLS in Abl and

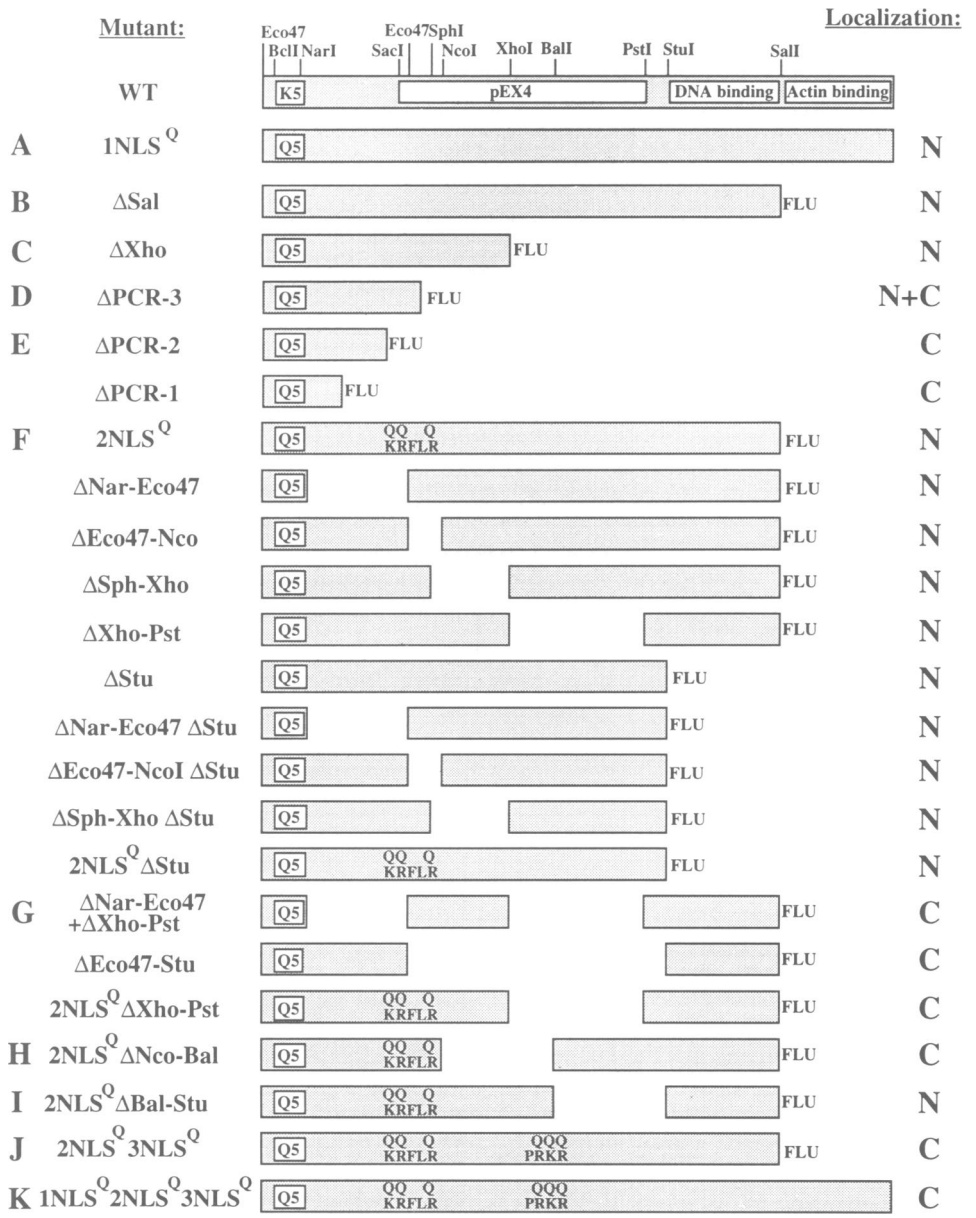


**Fig. 2.** Schematic representation of Abl mutants used to identify additional NLS. The myristoylated form (type IV) of murine c-Abl was utilized in all cases. Only the C-terminus of Abl, beginning at the end of the kinase domain, is illustrated. The positions of the first (K5) NLS, pEX4 region, DNA binding and actin binding domains, and unique restriction sites are shown. Except for constructs A and K, all proteins are truncated before the actin binding domain (ΔSal) or DNA binding domain (ΔStu), and epitope tagged (Mayer *et al.*, 1992) at the truncation site (to facilitate localization of the mutant protein) with a single copy of the influenza hemagglutinin peptide sequence (FLU) recognized by mAb 12CA5 (Wilson *et al.*, 1984). All constructs also contained a kinase-inactivating mutation (K290M) to improve protein expression; loss of kinase activity was shown previously not to affect subcellular localization of c-Abl (Van Etten *et al.*, 1994). Q5 indicates site-specific mutation of the first NLS, replacing each lysine residue with glutamine; site-specific mutations (glutamine for lysine or arginine) in the second ($2NLS^Q$) and third ($3NLS^Q$) Abl NLS are indicated.

to determine their approximate position, we fused portions of Abl to pyruvate kinase (PK) (Kalderon *et al.*, 1984; Dingwall *et al.*, 1988), and determined the localization of PK–Abl fusion proteins by immunofluorescence with anti-PK antibodies after transient expression in 293T cells. PK itself is wholly cytoplasmic (Figure 1A). Because of the loss of nuclear localization of Abl upon deletion of SH3 (Van Etten *et al.*, 1989), we wished to confirm that the SH3 domain itself had no nuclear localizing properties; a PK–SH3 fusion protein was also wholly cytoplasmic (Figure 1B). In contrast, a fusion protein of PK with the Abl C-terminus downstream of the first (K5) NLS localized to both nucleus and cytoplasm (Figure 1C), indicating the presence of one or more signals in this region of Abl capable of directing nuclear localization. To confirm this observation, we prepared glutathione *S*-transferase (GST)–Abl fusion proteins containing different C-terminal regions of Abl, microinjected them into 3T3 cells, and localized them by immunofluorescence with anti-GST antibodies. A GST fusion protein denoted GST–pEX4, containing Abl amino acids 726–866 (Figure 2), localized to the nucleus after microinjection (Figure 1D), while the parental GST protein (Figure 1E) and GST fusion proteins of other Abl C-terminal regions, including the DNA binding and actin binding domains (Van Etten *et al.*, 1994; data not shown), were cytoplasmic.

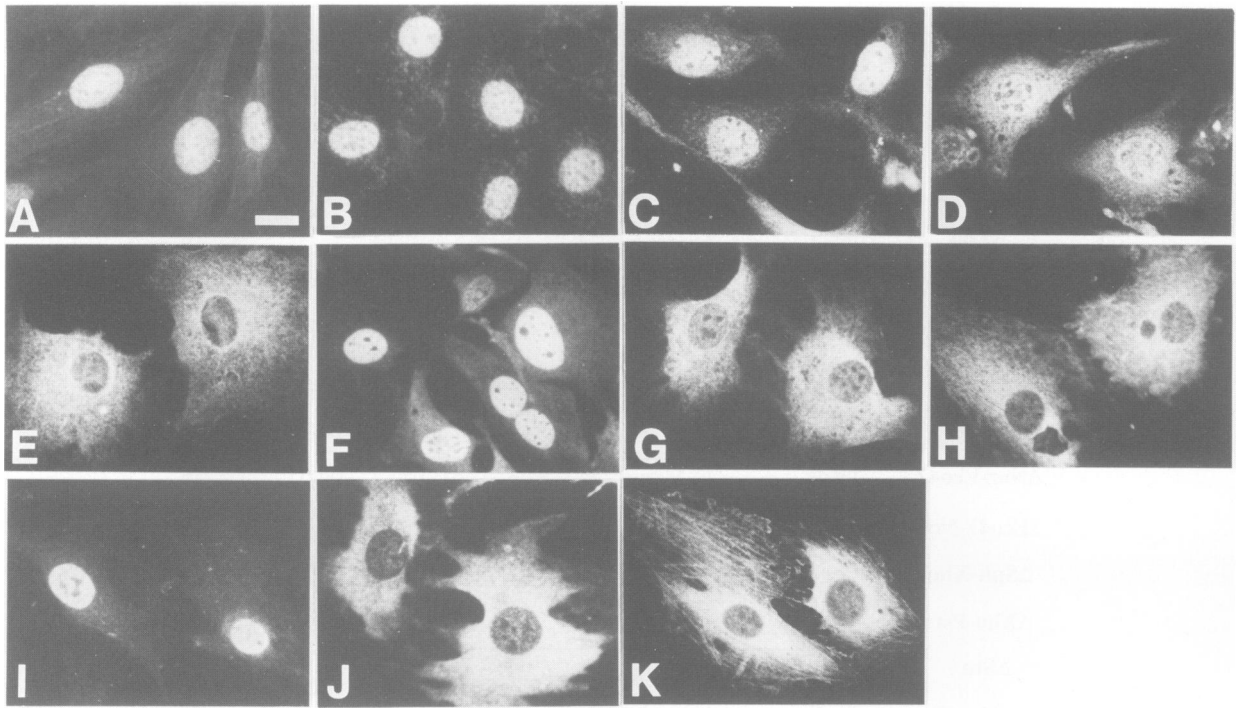


**Fig. 3.** Immunofluorescence analysis of localization of Abl mutant proteins. Abl mutant proteins depicted in Figure 2 were stably expressed in NIH 3T3 cells and localized by immunofluorescence; the localization of selected mutants (denoted by letters in Figure 2) is shown. Bar = 10 μm.

### *Two conserved basic motifs constitute independent Abl NLS*

To identify precisely additional Abl NLS, a series of Abl C-terminal truncation mutants with the pentalysine NLS mutated to pentaglutamine (Q5) were analyzed (Figure 2). To facilitate localization of mutant proteins without interference from endogenous c-Abl, the proteins were truncated before the actin binding domain (at the unique *Sal*I site) and epitope tagged at the C-terminus (Mayer *et al.*, 1992) with a single copy of the influenza hemagglutinin peptide recognized by the monoclonal antibody (mAb) 12CA5 (Wilson *et al.*, 1984). Epitope-tagged Abl protein with the Q5 mutation and truncation at the *Sal*I site, denoted c4 1NLS$^{Q}$ ΔSal FLU, was still nuclear (Figures 2B and 3B), as was a similar protein truncated at the *Xho*I site (Figures 2C and 3C), suggesting the presence of an NLS between the *Nar*I and *Xho*I sites in the c-Abl cDNA. Because there are no convenient restriction sites for further truncation in this interval, PCR was employed to make three further truncations, dividing this interval approximately into quarters. The longest PCR truncation mutant, ΔPCR-3 (Figure 2D), exhibited a mixed nuclear and cytoplasmic localization (Figure 3D), while the next shorter truncation, ΔPCR-2 (Figure 2E), was wholly cytoplasmic (Figure 3E). This suggested that the Abl second NLS was at or near the truncation defined by ΔPCR-3.

A short motif of basic amino acids (KRFLR), reminiscent of the SV40 (Kalderon *et al.*, 1984) and first Abl NLS, is bisected by the ΔPCR-3 truncation, suggesting that this sequence might be the second Abl NLS. However, when a site-specific mutation of this motif was generated, changing each basic lysine or arginine residue to glutamine, the resulting protein, c4 1NLS$^{Q}$ 2NLS$^{Q}$ ΔSal FLU (Figure 2F), was still nuclear in 3T3 cells (Figure 3F). A series of internal deletion mutations of Abl between the first NLS and the DNA binding domain, either alone or in combination with truncation of the DNA binding domain itself (Figure 2), also yielded Abl proteins which were localized to the nucleus (data not shown), raising the possibility of a third Abl NLS. Indeed, a mutant Abl protein with in-frame deletions in two discrete regions (c4 1NLS$^{Q}$ ΔNar-Eco47+ΔXho-Pst ΔSal FLU, Figure 2G) was localized exclusively to the cytoplasm (Figure 3G), indicating that the third NLS was located between the *Xho*I and *Pst*I sites. Further deletions with both the first and second NLS mutated suggested that the third NLS was proximal to the *Bal*I site, because the protein c4 1NLS$^{Q}$ 2NLS$^{Q}$ ΔNco-Bal ΔSal FLU (Figure 2H) was cytoplasmic (Figure 3H) while c4 1NLS$^{Q}$ 2NLS$^{Q}$ ΔBal-Stu ΔSal FLU (Figure 2I) was nuclear (Figure 3I). These results implicated another short basic motif (PRKR) as the third Abl NLS. Abl with all three NLS mutated (c4 1NLS$^{Q}$ 2NLS$^{Q}$ 3NLS$^{Q}$ ΔSal FLU, Figure 2J) was completely cytoplasmic (Figure 3J) and, with the C-terminal actin binding domain restored, became almost exclusively localized to the cytoskeleton (Figures 2K and 3K).

Both the second and third Abl NLS are absolutely conserved between mouse and human c-Abl (Figure 4), but are not conserved in the *Drosophila* Abl homolog. Interestingly, the three NLS are partially conserved in the *abl*-related gene *arg* (Kruh *et al.*, 1990), raising the possibility that Arg, like Abl, might be localized to the nucleus.

### *Cytoplasmic localization alone is insufficient to activate transformation by Abl*

Because transforming Abl proteins are exclusively cytoplasmic when localized by immunofluorescence, it is

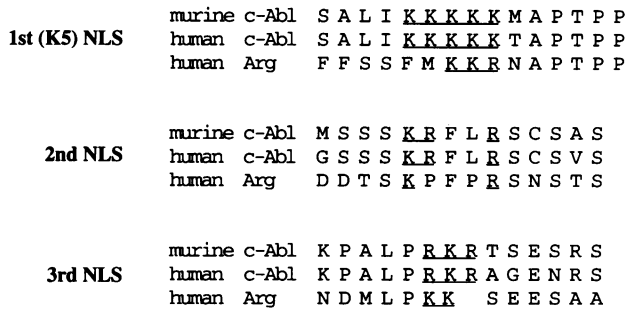


**Fig. 4.** Conservation of the three NLS between murine and human c-Abl and human Arg. The single letter amino acid code is employed; conserved basic residues are underlined.

possible that restricting c-Abl to the cytoplasm by mutation of the NLS might be transforming. We assessed the transforming properties of Abl proteins by focus formation after retroviral infection of NIH 3T3 cells (Table I). SH3-deleted Abl (c4 ΔXB) reproducibly yields $>10^4$ foci/ml, while an activated SH3 point mutant, P131L, is slightly lower. Focus formation is dependent on Abl kinase activity, as no foci are observed with a kinase-inactive Abl mutant (c4 ΔXB K290M). Truncation of the Abl C-terminus at the *BclI* site, with loss of all three NLS, reduced the focus-forming activity of SH3-deleted Abl by ~100-fold, while site-specific mutation of the three NLS similarly reduced transformation by the SH3 point mutant c4 P131L by ~10-fold. Therefore, nuclear localization is not required for transformation by activated Abl, but may increase the efficiency of transformation. In contrast, wild-type c-Abl reproducibly yielded a very low level of focus formation after retroviral infection with foci which are smaller in size and appear 1–2 days later than those observed with SH3-deleted Abl. This low level of focus-forming activity probably represents generation of activating mutations in the proviral DNA after reverse transcription, as no foci are observed when c-*abl* is introduced into NIH 3T3 cells by calcium phosphate transfection (Jackson and Baltimore, 1989; Jackson *et al.*, 1993b). Significantly, neither truncation of the C-terminus nor mutation of any combination of the three NLS was able to activate transformation by Abl (Table I). This demonstrates that cytoplasmic localization of Abl *per se* is not sufficient to induce transformation, but additional activating mutations, perhaps to release Abl kinase activity from a cellular inhibitor (Pendergast *et al.*, 1991; Van Etten *et al.*, 1995), are needed. Others have reported that a series of internal deletions of the c-Abl C-terminus can activate transformation by c-Abl independent of SH3 mutation (Goga *et al.*, 1993); these deletions include the second and third Abl NLS. However, site-specific mutation of these signals (c4 $2NLS^Q$ $3NLS^Q$) does not activate Abl, suggesting that loss of other Abl determinants in this region must be involved in the mechanism of activation of Abl in these mutants.

### *c-Abl and transforming Abl proteins inhibit entry into S phase, and growth inhibition requires nuclear localization*

To test the hypothesis that nuclear localization is important for the growth-inhibitory effect of Abl, we developed a quantitative transfection assay to measure inhibition of

**Table I.** Focus-forming ability of c-Abl NLS mutants

| Construct[a] | Titer (foci/ml)[b] in recipient cells[c] | |
|---|---|---|
| | NIH 3T3 | Clone 4A2+ |
| c4 ΔXB | $6.0\times10^4$ | $2.4\times10^3$ |
| c4 ΔXB ΔBcl | $6.4\times10^2$ | $9.0\times10^2$ |
| c4 ΔXB K290M | 0 | 0 |
| c4 P131L | $4.9\times10^3$ | $8.4\times10^2$ |
| c4 P131L $1NLS^Q$ $2NLS^Q$ $3NLS^Q$ | $5.3\times10^2$ | $7.4\times10^2$ |
| c4 | 30 | |
| c4 K290M | 0 | |
| c4 ΔBcl | 11 | |
| c4 $2NLS^Q$ | 9 | |
| c4 $3NLS^Q$ | 33 | |
| c4 $2NLS^Q$ $3NLS^Q$ | 15 | |
| c4 $1NLS^Q$ | 12 | |
| c4 $1NLS^Q$ $2NLS^Q$ | 8 | |
| c4 $1NLS^Q$ $3NLS^Q$ | 25 | |
| c4 $1NLS^Q$ $2NLS^Q$ $3NLS^Q$ | 5 | |

[a]K290M is a kinase-inactivating mutation, ΔXB is an SH3 deletion of c-Abl and P131L is an SH3 point mutation. Site-specific mutations in the first, second and third Abl NLS are denoted by $1NLS^Q$, $2NLS^Q$ and $3NLS^Q$ respectively.
[b]Data from independent infection experiments, normalized to a proviral titer of 0.25 copy/cell as described in Materials and methods.
[c]SH3-mutated Abl viruses were titered on a standard laboratory stock of NIH 3T3 cells or the permissive NIH 3T3 clone 4A2+ (Renshaw *et al.*, 1988).

$G_1$–S progression by Abl. NIH 3T3 cells are transfected with Abl and *lacZ* expression plasmids and subsequent progression of transfected cells through S phase monitored by nuclear incorporation of 5-bromo-2-deoxyuridine (BrdU) (Figure 5A). About 40–60% of cells transfected with *lacZ* alone or kinase-inactive c-Abl stained positively for BrdU incorporation after 40 h, indicating passage through S phase of the cell cycle. In contrast, when kinase-active c-Abl was transfected, there was a reproducible 4- to 5-fold decrease in the percentage of transfected cells which incorporated BrdU, indicating a retardation or block in entry into S phase (Figure 5B). In addition to this cytostatic effect, a consistent reduction in transfection efficiency was observed with kinase-active c-Abl, coincident with the appearance of frequent pyknotic cells, suggesting that the expression of this protein was also acutely lethal to some recipient cells. Site-specific mutation of the three Abl NLS abolished the growth-inhibitory effect and increased transfection efficiency. Addition of a peptide containing the minimal functional SV40 large T antigen NLS ($PKK_{128}KRKV$) (Kalderon *et al.*, 1984) to the C-terminus of c-Abl lacking all three endogenous NLS restored the cytostatic effect (Figure 5B) and nuclear localization (data not shown), while a mutant peptide ($PKT_{128}KRKV$) did not. These results demonstrate that nuclear localization is absolutely required for growth inhibition by Abl.

In addition to c-Abl, the transforming c-Abl proteins c4 ΔXB (Jackson and Baltimore, 1989), which contains a deletion of SH3, and c4 P131L (Van Etten *et al.*, 1995), which contains an activating point mutation in SH3, also exhibited a prominent cytostatic effect in NIH 3T3 cells which was likewise absolutely dependent on nuclear localization (Figure 5B). Although these transforming Abl proteins are cytoplasmic when localized by immunofluo-

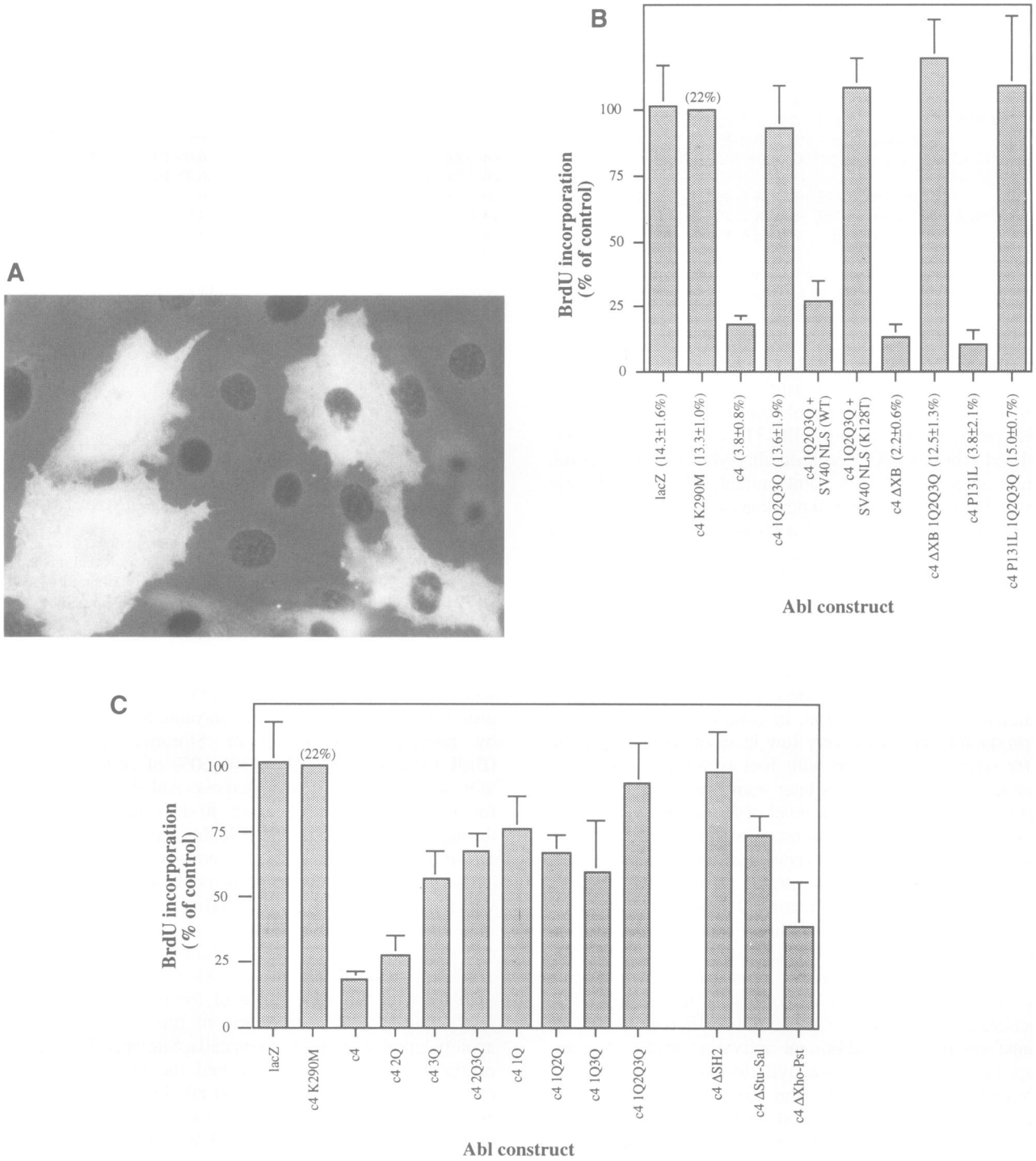


**Fig. 5.** c-Abl and transforming Abl are cytostatic, and nuclear localization is required. (**A**) Photomicrograph of NIH 3T3 cells transfected with a cytostatic allele of Abl. In this field, four transfected cells are detected by cytoplasmic β-galactosidase immunofluorescence staining, while passage of the two cells on the right through S phase is indicated by nuclear BrdU histochemical staining. The cells were photographed under epifluorescence illumination with a low level of transillumination sufficient to detect the nuclear BrdU histochemical stain. (**B**) Inhibition of S phase entry by Abl proteins, measured as the percentage of transfected cells positive for BrdU incorporation relative to kinase-inactive Abl. The average of several experiments ($n \geq 3$) is shown. In Figures 5 and 6, the absolute percentage of BrdU-positive cells transfected with kinase-inactive Abl (c4 K290M) is shown in parentheses above the bar for this construct. The average transfection efficiency for each construct is shown in parentheses following the construct name. Nomenclature: lacZ denotes transfection of the β-galactosidase reporter construct alone, c4 denotes c-Abl type IV, K290M is a kinase-inactivating point mutation, ΔXB is an SH3 deletion of c-Abl (Jackson and Baltimore, 1989), P131L is an activated SH3 point mutant of c-Abl (Van Etten *et al.*, 1995) and $1^Q$, $2^Q$ and $3^Q$ refer to site-specific mutants in the first, second and third Abl NLS, respectively. All proteins were expressed and found to be of the expected molecular weight in the transfected cells by Western blot (data not shown). By immunofluorescence analysis of the same cells, c4 and c4 K290M were largely nuclear, while c4 $1^Q2^Q3^Q$ was exclusively cytoplasmic; the transforming proteins c4 ΔXB, c4 ΔXB $1^Q2^Q3^Q$, c4 P131L and c4 P131L $1^Q2^Q3^Q$ were cytoplasmic, as previously reported (Van Etten *et al.*, 1995). (**C**) The three Abl NLS contribute independently to the cytostatic effect, and the Abl SH2 and DNA binding domains are also required.

rescence, it is possible that a small fraction may be nuclear, and in combination with deregulation of the kinase activity may induce a similar cytostatic effect to that of c-Abl. The v-Abl proteins P160 and P120 [a naturally occurring deletion mutant of P160 lacking all three NLS (Reddy *et al.*, 1983)] exhibited intermediate growth-suppressive activity which was not affected by mutation of the three NLS in P160 (data not shown), suggesting that Gag–Abl proteins may gain access to the nucleus via other signals or inhibit growth through other mechanisms, perhaps as a consequence of their greatly increased kinase activity. We also found the P210 Bcr–Abl protein of human chronic myelogenous leukemia to have a moderate cytostatic effect (data not shown).

### *The three Abl NLS independently contribute to the cytostatic effect*

The three NLS of Abl contribute to the cytostatic effect in an additive manner, with all three signals required for maximum growth inhibition (Figure 5C). While mutation of the second NLS alone had little effect on toxicity, mutation of the third Abl NLS eliminated over half of the cytostatic effect (Figure 5C, construct $3^{Q}$). Similarly, mutation of the first NLS alone eliminated over half the cytostatic effect, but growth inhibition was not abolished until all three NLS were mutated (Figure 5C, compare construct $1^{Q}$ with $1^{Q}2^{Q}3^{Q}$). We also examined whether other functional domains of Abl are required for the cytostatic effect. The previous results indicated that tyrosine kinase activity was absolutely required, while the SH3 domain was not. Interestingly, the SH2 domain appeared to be required for the growth-inhibitory effect (Figure 5C, construct c4 ΔSH2). Deletion of the Abl DNA binding domain also greatly diminished the cytostatic effect (Figure 5C, construct c4 ΔStu-Sal), while deletion of an adjacent C-terminal region had much less effect on growth inhibition (Figure 5C, construct c4 ΔXho-Pst).

### *p53 and Rb tumor suppressor gene products are required for cell cycle inhibition by Abl*

To investigate the cellular requirements for the Abl cytostatic effect, we utilized primary embryo fibroblasts derived from mice with homozygous null mutations in the *Rb* and *p53* tumor suppressor genes (Jacks *et al.*, 1992; Lowe *et al.*, 1993) and their wild-type littermates, which are genetically identical except for the presence or absence of the *Rb* or *p53* gene product, respectively. c-Abl and transforming Abl proteins were strongly cytostatic in $Rb^{+/+}$ fibroblasts, dependent on SH2 and nuclear localization (Figure 6A, left). In contrast, there was no detectable cytostatic effect of Abl in $Rb^{-/-}$ fibroblasts (Figure 6A, right), demonstrating that the Rb protein is absolutely required for growth inhibition by Abl. The growth-inhibitory effect of Abl was substantially restored to $Rb^{-/-}$ fibroblasts by co-transfection of an Rb expression plasmid, but not by co-expression of a mutant form of Rb with a deletion in the A/B pocket region (Figure 6A, far right). In a similar experiment utilizing cells from *p53* mutant mice, Abl was cytostatic in $p53^{+/+}$ fibroblasts but not in $p53^{-/-}$ fibroblasts (Figure 6B), indicating that *p53* is also required for this effect. We also examined whether Abl would be cytostatic in clones of NIH 3T3 cells which had been isolated originally for displaying growth arrest or growth stimulation in response to the v-Abl oncogene (Renshaw *et al.*, 1992). Interestingly, Abl was cytostatic in non-permissive (N) NIH 3T3 cells, which exhibit growth arrest in response to v-Abl, but not in permissive (P) NIH 3T3 cells, which are growth stimulated by v-Abl (Figure 6C). The precise biochemical difference between N- and P-3T3 cells is not understood; the two cell types exhibit modulation of alternative $G_1$ cyclin/cdk activities in response to phorbol ester, but do not differ in *p53* or *Rb* status (Huang *et al.*, 1995).

To confirm the requirement for *Rb* and *p53*, we repeated the cytostasis assay in NIH 3T3 cells under conditions where the function of the endogenous *Rb* or *p53* gene products was inactivated. The E7 gene product of high risk types of human papilloma virus (HPV) binds and inactivates p110 Rb, as well as the Rb-related proteins p107 and p130 (Munger *et al.*, 1989), while the HPV E6 gene product binds and inactivates p53 by targeting it for ubiquitination and degradation (Scheffner *et al.*, 1990). We co-transfected NIH 3T3 cells with Abl and expression constructs for HPV-16 E7 or E6, and observed a striking loss of the Abl-induced cytostatic effect with co-expression of either HPV oncoprotein (Figure 6D); importantly, the level of BrdU incorporation into 3T3 cells transfected with kinase-inactive Abl and E6 or E7 was similar to previous experiments, indicating that E6 or E7 did not stimulate DNA synthesis significantly under these conditions. We also co-transfected NIH 3T3 cells with Abl and the temperature-sensitive V135 mutant of p53 (Michalovitz *et al.*, 1990), which behaves as wild-type at 32°C but acts as a dominant-negative at 37°C, and observed a prominent cytostatic effect at 32°C but not at 37°C (Figure 6E). A similar loss of the Abl cytostatic effect was seen with co-transfection of NIH 3T3 cells with an expression plasmid encoding adenovirus E1A (which binds Rb) or the 55 kDa form of E1B (which binds p53) (data not shown). These results provide an independent demonstration of the requirement for Rb and p53, and suggest that Abl blocks cell growth at the $G_1$–S transition by interacting with central elements of the cell cycle control apparatus in the nucleus.

## Discussion

We have identified two additional short sequence motifs in the Abl C-terminus, both of which can act in a necessary and sufficient manner to localize c-Abl to the nucleus. Abl is unique among nuclear proteins in having three independent NLS. Bipartite NLS have been defined in several proteins, including nucleoplasmin (Robbins *et al.*, 1991), Rb (Zacksenhaus *et al.*, 1993) and p53 (Addison *et al.*, 1990) (for review, see Dingwall and Laskey, 1991). These signals typically consist of two clusters of basic amino acids separated by a short spacer of 10–12 residues, with both parts required for activity. The three Abl NLS are widely separated and function independently, and therefore do not fit the bipartite pattern. The first Abl NLS (which has the sequence RDRK $N_8$ KKKKK) may itself be of this class, but the requirement for the N-terminal basic motif has not been established. The second and third Abl NLS are short basic motifs similar to the SV40 large T antigen NLS; the third Abl NLS (PRKR) is most like the adenovirus E1A NLS (KRPRP) (Lyons *et al.*, 1987).

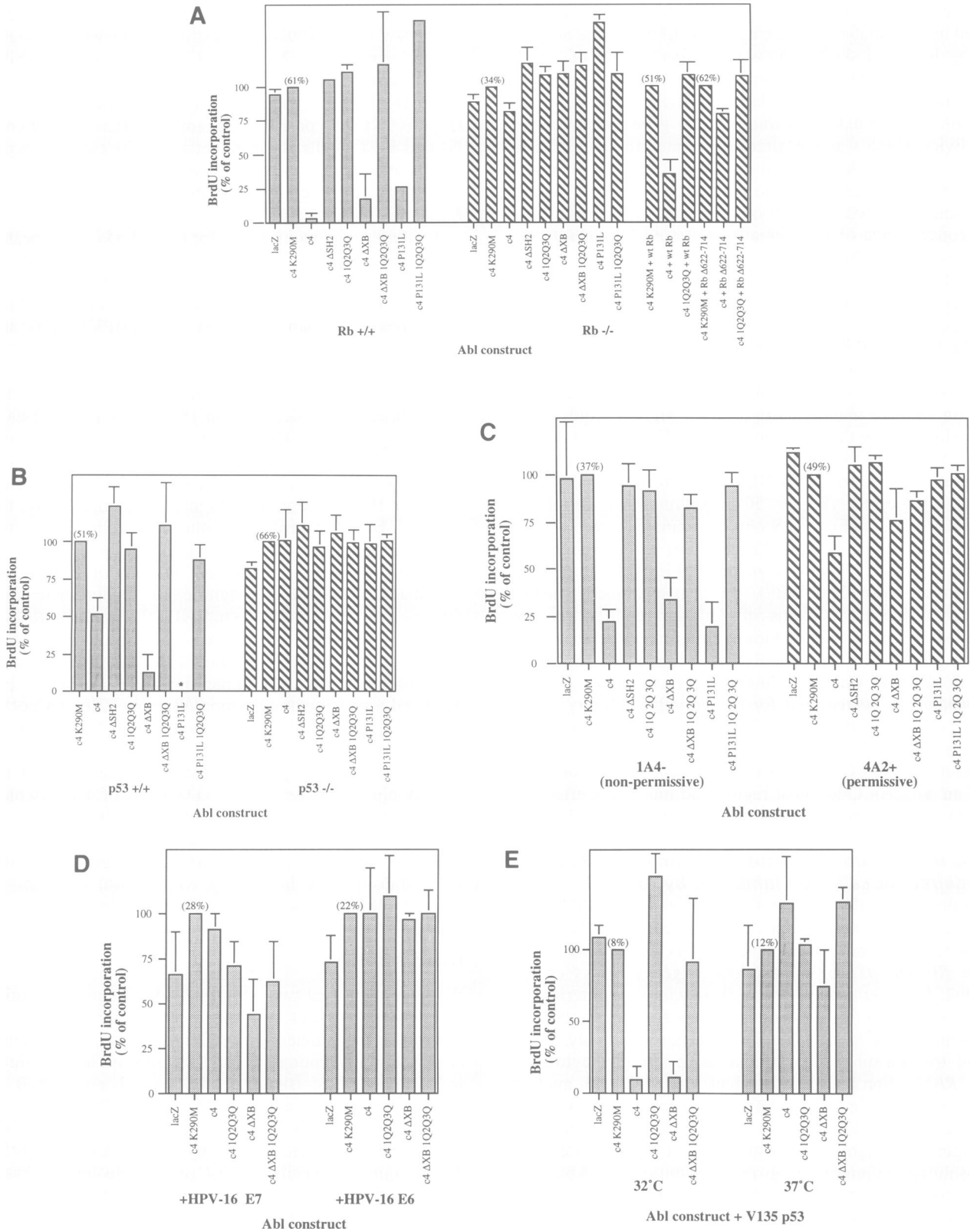


**Fig. 6.** The *Rb* and *p53* tumor suppressor gene products are required for cell cycle inhibition by Abl. (**A**) The transfection assay for Abl cytostatic effect was performed in primary embryo fibroblasts derived from mice with a homozygous null mutation in the *Rb-1* locus ($Rb^{-/-}$) or wild-type littermates ($Rb^{+/+}$) (Jacks *et al.*, 1992). (**B**) Same as in (A), except that cells were from *p53*-deficient mice ($p53^{-/-}$) or wild-type littermates ($p53^{+/+}$) (Lowe *et al.*, 1993). In multiple experiments we were unable to detect viable $p53^{+/+}$ cells expressing the c4 P131L construct (asterisk). (**C**) The cytostasis assay was performed in clones of NIH 3T3 cells which exhibit growth arrest (non-permissive, clone 1A4–) or growth stimulation (permissive, clone 4A2+) in response to v-Abl (Renshaw *et al.*, 1992). (**D**) The cytostasis assay was performed in NIH 3T3 cells with the addition of an expression plasmid encoding the HPV-16 E7 or E6 gene. (**E**) The cytostasis assay was performed in NIH 3T3 cells with the addition of an expression plasmid encoding the temperature-sensitive V135 mutant of murine p53 (Michalovitz *et al.*, 1990); following transfection, cells were maintained at 32 or 37°C prior to fixation.

Several proteins have been found to have two NLS (Richardson *et al.*, 1986; Hall *et al.*, 1990; Underwood and Fried, 1990) whose function is interdependent or hierarchical in nature. A notable exception is the muscle-specific transcription factor MyoD, which has two adjacent signals which appear to function completely independently of one another (Vandromme *et al.*, 1995).

Deletion mutagenesis can reveal the presence of 'masked' or cryptic NLS which function in a mutant protein but not in the context of the wild-type protein. For example, the glucocorticoid receptor has a hormone-inducible NLS which functions under normal physiological conditions, and a second signal which becomes constitutively active only after the hormone binding domain is deleted (Picard and Yamamoto, 1987). Several lines of evidence suggest that the three Abl NLS are not artifacts of mutagenesis. First, the three signals are absolutely conserved between mouse and human Abl, albeit in regions with ~80% similarity at the amino acid level. Importantly, all three Abl NLS function independently of one another in the context of the full-length protein to localize Abl to the nucleus. Finally, all three signals have been implicated in regulating the cytostatic phenotype of Abl.

Why would Abl require three independent signals for nuclear localization? A likely possibility is that the three signals are regulated differently. The second and third Abl NLS function in fibroblasts, but not in COS cells (Van Etten *et al.*, 1989), demonstrating that the three signals function differently in distinct cell types. The first Abl NLS may be regulated by adjacent protein kinase C (Pendergast *et al.*, 1987) and cdc-2 kinase (Kipreos and Wang, 1990) phosphorylation sites, while the second and third NLS have no obvious nearby phosphorylation sites. Further, while the first (pentalysine) NLS will localize Abl preferentially to the nucleus even in the presence of the actin binding domain, the second or third Abl NLS alone is significantly less efficient at inducing nuclear localization when the actin binding domain is present, with the majority of the protein associated with the cytoskeleton (data not shown). Together, these results suggest a model where Abl may move from place to place in the cell (for example, from cytoskeleton to nucleus) in response to different physiologic stimuli. Synchronization has not revealed a simple change in the localization of overexpressed c-Abl during the cell cycle, except for an exclusion of Abl from chromatin during M phase (R.A.Van Etten, unpublished data); however, the movement of a fraction of Abl from one compartment to another would not be detected in these experiments. Site-specific mutations in the NLS, the cytoskeletal association domain and their regulatory elements may allow the intracellular trafficking pattern of Abl to be elucidated.

Our results establish that Abl must be in the nucleus to exert its negative effect on cell proliferation. A major question is whether the cytotoxic/cytostatic effect of Abl, observed after transfection, reflects a physiological role of Abl or is instead a toxic manifestation of artificial overexpression of the protein. One possibility is that growth suppression by Abl is a consequence of over-expressing a positive cell cycle regulator (Abl) inappropriately—perhaps the Abl kinase must be inhibited by Rb during $G_1$ to avoid toxicity. However, the requirement for Rb for Abl growth suppression is not consistent with this model, which would predict enhanced toxicity in the absence of Rb. In addition, expression of antisense c-*abl* sequences in NIH 3T3 cells leads to a reduction in the length of $G_1$ when quiescent cells are stimulated to enter the cell cycle, consistent with inhibition of the $G_1$–S transition by endogenous c-Abl (Daniel *et al.*, 1995). We therefore postulate that the Abl cytostatic effect represents a normal function of Abl, at least under some conditions.

c-Abl may be activated to transform fibroblasts by deletions or point mutations in the SH3 domain (Franz *et al.*, 1989; Jackson and Baltimore, 1989; Van Etten *et al.*, 1995), which may relieve Abl kinase activity from the action of a cellular inhibitor (Pendergast *et al.*, 1991; Van Etten *et al.*, 1995). We have demonstrated that SH3-mutated Abl proteins are also cytostatic. This appears paradoxical, because transforming c-Abl proteins are wholly cytoplasmic when localized by immunofluorescence (Van Etten *et al.*, 1989, 1995). However, the cytostatic/cytotoxic effect of SH3-mutated Abl is eliminated completely by mutation of the three NLS, demonstrating that nuclear localization is absolutely required. The discrepancy may be resolved if it is postulated that a small fraction of SH3-mutated Abl may be present in the nucleus. The change in localization of transforming Abl proteins from the nucleus to cytoplasm requires tyrosine phosphorylation and the Abl SH2 domain (Van Etten *et al.*, in preparation), implying that the function of the NLS may be inhibited when Abl is complexed with tyrosine-phosphorylated proteins. A fraction of SH3-deleted Abl may lack phosphotyrosine and remain in the nucleus, and mediate the growth-suppressive effect by virtue of its dysregulated tyrosine kinase activity. Because the cytostatic effect of c-Abl and SH3-mutated Abl both require nuclear localization (and the *Rb* and *p53* gene products), it is likely that they represent the same phenomenon. Restricting c-Abl to the cytoplasm by mutation of the three NLS was insufficient for transformation, indicating that additional activating mutations are required. In contrast, we found that mutation of the three NLS decreased the transforming potential of activated alleles of Abl, revealing that nuclear localization may play a positive role in cellular transformation by Abl. The transforming potential of activated Abl with or without functional NLS was similar in the permissive 4A2+ cells (Table I), suggesting that the signaling pathways utilized by nuclear Abl in transformation and growth arrest may overlap.

Several Abl proteins inhibit cell growth despite lacking all Abl NLS. The viral Gag–Abl proteins P160 and P120 v-Abl [which lacks all three NLS due to an in-frame C-terminal deletion of 263 amino acids (Reddy *et al.*, 1983)] are both associated with prominent cytotoxicity upon infection of fibroblasts with the appropriate Ab-MuLV strain (Ziegler *et al.*, 1981; Goff *et al.*, 1982). Recently, another potential NLS has been identified in P120 v-Abl which may confer nuclear localization in myeloid cells (Birchenall-Roberts *et al.*, 1995), but our results indicate that this putative signal does not function in 3T3 cells, because the constructs c4 $1NLS^Q$ $2NLS^Q$ ΔNco-Bal and c4 $1NLS^Q$ $2NLS^Q$ $3NLS^Q$ contain this signal but are wholly cytoplasmic (Figure 3H and J, respectively). In the cytostasis assay, both the P160 and P120 forms of v-Abl induced an intermediate level of growth suppression, and mutation of the three NLS in

P160 had no effect on growth-suppressive activity (data not shown). These results suggest that Gag–Abl proteins may gain access to the nucleus through determinants in the Gag polypeptide or via chaperone effects (Dingwall *et al.*, 1982), or that they suppress cell growth through other mechanisms. The fact that both v-Abl and c-Abl are cytostatic in 'non-permissive' NIH 3T3 cells but not in 'permissive' 3T3 cells (Renshaw *et al.*, 1992) suggests that the mechanism of growth suppression by the two forms of Abl may be the same. In addition, a fusion protein of the c-Abl N-terminus and kinase domain joined to the hormone binding domain of the estrogen receptor has been shown to transform fibroblasts in the presence of estradiol (Jackson *et al.*, 1993a) and arrest the same cells in $G_1$ in the absence of hormone (Mattioni *et al.*, 1995); although the majority of a cytostatic Abl–estrogen receptor fusion protein was located in the cytoplasm by immunofluorescence, significant levels of nuclear staining were also observed (Mattioni *et al.*, 1995). Understanding the mechanism of growth suppression by these and other variants of Abl will require careful analysis of subcellular localization, and identification and mutagenesis of all potential NLS.

The precise biochemical mechanism of growth inhibition by Abl remains to be determined. We find that Abl requires the presence of both the *Rb* and *p53* tumor suppressor gene products to inhibit cell proliferation. Using the instability of expression of cytostatic Abl proteins as a marker of growth inhibition, others recently have shown that this effect requires *p53* but not *Rb* (Goga *et al.*, 1995). The discrepancy with our results might be explained by the indirect nature of this assay, where factors other than cell cycle inhibition might decrease the stability of Abl expression in $Rb^{-/-}$ cells. p53 can induce cell cycle arrest following a variety of stimuli by induction of $p21^{WAF1/CIP1}$, which blocks cell growth by inhibition of a broad range of cyclin-dependent kinases (Dulic *et al.*, 1994) and also by direct inhibition of DNA replication via inactivation of proliferating cell nuclear antigen (PCNA; Waga *et al.*, 1994). Rb is postulated to inhibit cell proliferation in its unphosphorylated form by sequestering essential transcription factors of the E2F family (Nevins, 1992), and phosphorylation of Rb by D-type cyclins and cyclin-dependent kinase 4 or 6 releases E2F and promotes entry into S phase (Sherr, 1994). While no explicit connection between p53 and Rb has been demonstrated previously, it is suggested by the observations that p53-dependent $G_1$ arrest is defective in $Rb^{-/-}$ cells and abrogated by HPV E7 (Slebos *et al.*, 1994), and that E7 or E1A overcomes growth arrest in cells immortalized with the V135 temperature-sensitive p53 mutant (Vousden *et al.*, 1993).

Likewise, our results strongly imply that both p53 and Rb participate in the Abl growth suppression pathway, because the Abl cytostatic effect is lost in fibroblasts genetically deficient for either protein, and is abolished in NIH 3T3 cells when the function of the endogenous Rb or p53 proteins is blocked. This result is not due to lack of Abl nuclear localization from loss of a chaperone, because c-Abl is still localized to the nucleus in $p53^{-/-}$ and $Rb^{-/-}$ fibroblasts (data not shown). There is no further diminution of the Abl cytostatic effect in $Rb^{-/-}$ cells when endogenous p53 is also inactivated (data not shown), suggesting that p53 and Rb are involved in the same biochemical pathway, rather than acting in parallel. Although Abl kinase activity and the SH2 domain are required for growth inhibition, it is unlikely that either Rb or p53 is a direct substrate of Abl because neither protein is detectably tyrosine phosphorylated *in vivo*. It is difficult to test whether direct binding of Abl to Rb is necessary for growth suppression, because Abl binds to Rb through the kinase domain (Welch and Wang, 1993) and kinase activity is required for the cytostatic effect. Deletion of the Abl DNA binding domain caused a large decrease in growth-suppressive activity, but it is not clear whether it is DNA binding or other functions of Abl in this region which are required for growth arrest. Interestingly, DNA damage has been shown recently to stimulate c-Abl kinase activity and lead to activation of the stress kinase pathway (Kharbanda *et al.*, 1995). Together with our results, this suggests that Abl might play a role in the cell cycle response to DNA damage. We postulate that Abl acts upstream of p53, perhaps to relieve inhibition of p53 or induce or stabilize p53 function, and Rb is involved through the action of a cdk inhibitor such as p21. The Abl inhibitory effect may be mediated by p21 but occur before activation of cyclin E, and therefore act exclusively to block cyclin D/cdk4-dependent phosphorylation of Rb. Alternatively, p53 can inhibit cell growth through p21-independent mechanisms (Deng *et al.*, 1995; Hirano *et al.*, 1995) which may require Rb. The recent availability of primary mouse embryo fibroblasts deficient in p21 (Deng *et al.*, 1995) will allow direct testing of this model. The biochemical pathway of inhibition of S phase by Abl is now amenable to detailed analysis, and should provide further insight into the physiology of normal and transforming Abl proteins.

## Materials and methods

### Cells and cell culture

NIH 3T3 and 293T cells were the kind gift of Dr Lloyd Klickstein, Department of Rheumatology, Brigham & Women's Hospital, Boston MA. NIH 3T3 clones 1A4 and 4A2 (Renshaw *et al.*, 1992) were the kind gift of Dr Jean Wang, Department of Biology, University of California, San Diego CA. 3T3 lines were propagated in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, 10% calf serum and penicillin/streptomycin. Primary embryo fibroblasts from mice with homozygous mutations in the *Rb* (Jacks *et al.*, 1992) or *p53* (Lowe *et al.*, 1993) genes were the kind gift of Dr Tyler Jacks, Center for Cancer Research, Department of Biology, MIT, Cambridge MA, and were propagated in DMEM with 10% heat-inactivated fetal calf serum and penicillin/streptomycin. All experiments on primary embryo fibroblasts were carried out on cells which had been passed fewer than five times in culture. Cells were cultured in a humidified atmosphere containing 5% $CO_2$.

### DNA constructs

Mutagenic analysis of the Abl NLS was carried out in the retroviral expression vector pPL (Van Etten *et al.*, 1989), utilizing a murine type IV c-*abl* cDNA containing a kinase-inactivating point mutation (K290M) to improve expression. This mutation was shown previously not to alter the subcellular localization of Abl in 3T3 cells (Van Etten *et al.*, 1994). The construct also contained a site-specific mutation in the pentalysine (K5) NLS, with alteration to pentaglutamine (Q5) (Van Etten *et al.*, 1989). In addition, to allow specific detection of mutant Abl proteins, the c-*abl* reading frame was truncated at the *Sal*I site (at the beginning of the Abl actin binding domain) and epitope tagged tagged at the C-terminus (Mayer *et al.*, 1992) with a single copy of the influenza hemagglutinin epitope (YPYDVPDYA) recognized by the mAb 12CA5 (Wilson *et al.*, 1984). This DNA, denoted pPL c4 K290M Q5 ΔSal FLU, served as the starting point for further mutagenesis.

PCR mutagenesis utilized 3′ primers containing a *Sal*I recognition site and homology to the *abl* sequence ending at nucleotide 1975, 2071 or 2185 (c-*abl* numbering according to Oppi *et al.*, 1987) in conjunction with a common 5′ primer derived from sequences upstream of the unique *Nar*I site at nucleotide 1879. PCR products were digested with *Nar*I and *Sal*I, gel-purified, and cloned into pPL c4 K290M Q5 ΔSal FLU to generate the truncation mutants ΔPCR-1, ΔPCR-2 and ΔPCR-3, respectively. Two independent clones of each PCR mutant were expressed in each case, with identical results. Internal deletion mutants were constructed by digestion with the indicated restriction enzymes, generation of appropriate blunt ends by T4 DNA polymerase or mung bean nuclease, and fusion of the sequence in-frame by blunt-end ligation with T4 DNA ligase. Site-specific mutagenesis of the second and third NLS was performed utilizing the *dut/ung* phagemid mutagenesis system (Kunkel, 1985) (Bio-Rad Laboratories) as described (Mayer *et al.*, 1992). Addition of wild-type ($PKK_{128}KRKV$) or mutant ($PKT_{128}KRKV$) SV40 NLS peptides to the C-terminus of Abl was done by synthesis of complementary oligonucleotides encoding the appropriate peptide and cloning into *Sal*I and *Hin*dIII sites at the 3′ end of the *abl* cDNA which were generated by PCR. The sequence of each fusion junction and site-specific mutant was verified by DNA sequencing. For assay of cell cycle arrest in transiently transfected NIH 3T3 cells or primary mouse embryo fibroblasts, type IV c-*abl* parental or mutant cDNAs (lacking an epitope tag) were cloned into the expression vector pcDNA I/Amp (InVitrogen).

cDNAs encoding the E6 and E7 genes from the high risk HPV strain HPV-16, in the eukaryotic expression vector pRSV (Munger *et al.*, 1989) were the generous gift of Dr Karl Münger, Department of Pathology, Harvard Medical School. The ts V135 mutant of murine p53 in the eukaryotic expression vector pLTRp53cGval135 (Michalovitz *et al.*, 1990) was the kind gift of Dr Phil Hinds, Department of Pathology, Harvard Medical School. cDNAs encoding human pRb and a PCR-generated Rb mutant carrying an in-frame deletion removing amino acids 622–714 (Hu *et al.*, 1990) were kindly provided by Dr Ed Harlow, Massachusetts General Hospital Cancer Center, and were subconed into the pcDNA expression vector prior to use.

Pyruvate kinase fusion constructs utilized the vector p3PK (Frangioni and Neel, 1993), with Abl sequences fused in-frame to the C-terminus of PK. Abl sequences consisted of nucleotides 209–408 (*Xmn*I–*Hin*cII) encompassing the SH3 domain, and nucleotides 1883–2928 (*Nar*I–*Sal*I) encompassing the Abl C-terminus between the first NLS and actin binding domain.

### *Transfections*

NIH 3T3 cells stably expressing epitope-tagged Abl protein were isolated by calcium phosphate co-transfection with pSV2*neo*, selection for neomycin resistance and screening individual clones by indirect immunofluorescence as described (Van Etten *et al.*, 1995) with mAb 12CA5. The localization of each mutant protein was confirmed by examination of multiple independent clones, and expression of epitope-tagged Abl protein of the predicted molecular weight was confirmed in each case by Western blotting (data not shown).

For analysis of cell cycle arrest by Abl mutants, cells were plated at $1.5–2.0\times10^5$ cells per plate on glass coverslips in 3.5 cm tissue culture plates the day before transfection. Cells were transfected with 0.5 μg of pcDNA/lacZ reporter plasmid and 1.5 μg of *abl* mutant in the pcDNA expression vector, along with 6 μl of LipofectAMINE reagent (2 mg/ml, Gibco/Life Technologies) in a volume of 1 ml of serum-free medium per plate. After incubation for 5 h at 37°C, the medium was aspirated and fresh medium containing 10% calf serum added. Twenty-one hours post-transfection, 5-bromo-2-deoxyuridine (BrdU, Sigma) at 10 μM was added to the medium, and coverslips fixed and stained 36–40 h post-transfection as described below. In experiments where other genes were co-transfected with *abl*, a mixture of 0.75 μg of *abl* plasmid and 0.75 μg of the other expression plasmid was utilized.

Calcium phosphate transfection of 293T cells was as described (Van Etten *et al.*, 1995). For analysis of focus-forming activity of Abl mutants, *abl* mutant cDNAs in the pPL vector were transfected into the packaging cell line BOSC-23 (Pear *et al.*, 1993), viral supernatants harvested at 48 h and used to infect NIH 3T3 cells as described (Van Etten *et al.*, 1995). Foci were scored at 10 days post-infection and normalized to a proviral copy number of 0.25 per cell, determined by Southern blot analysis of genomic DNA derived from a parallel infected plate.

### *Immunofluorescence and histochemical staining*

For immunofluorescence localization of epitope-tagged Abl proteins, cells were fixed in methanol and acetone as described (Van Etten *et al.*, 1989) and treated with primary 12CA5 mAb (protein A-purified, 10 μg/ml, BabCo) followed by rhodamine-conjugated donkey anti-mouse IgG (10 μg/ml, Jackson Immunoresearch). To assess cell cycle arrest, dual immunofluorescence and histochemical staining was performed. Cells were fixed in acid alcohol (5% acetic acid, 95% ethanol) at room temperature for 30 min, treated with polyclonal rabbit anti-β-galactosidase antibodies (Cappell) and rhodamine-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch), followed by histochemical detection of BrdU incorporation utilizing the Cell Proliferation Kit (Amersham); the primary antibody was mouse anti-BrdU and secondary antibody anti-mouse horseradish peroxidase conjugate. Color was developed with DAB/cobalt enhancement.

GST fusion proteins were generated utilizing the Glutagene System (Pharmacia) and purified from lysates of *Escherichia coli* as described (Van Etten *et al.*, 1989). Purified GST fusion proteins were microinjected into NIH 3T3 cells and localized by immunofluorescence with affinity-purified anti-GST antibodies as described (Van Etten *et al.*, 1994).

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