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The Mouse Type IV c-*abl* Gene Product Is a Nuclear Protein, and Activation of Transforming Ability Is Associated with Cytoplasmic Localization

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Summary

The subcellular localization of the mouse type IV c-*abl* protein was determined by indirect immunofluorescence of nontransformed NIH 3T3 fibroblasts that overexpress the protein. Unlike the viral transforming protein p160$^{gag/v\text{-}abl}$, which has cytoplasmic and plasma membrane localization, a large fraction of the c-*abl* (IV) protein is nuclear, with the remainder in the cytoplasm and plasma membrane. Deletion of a small N-terminal regulatory region of the c-*abl* (IV) protein, sufficient to activate its transforming potential fully, changes the distribution of the protein from the nucleus to the cytoplasm. Mapping of an amino acid sequence responsible for the nuclear localization of the c-*abl* (IV) protein reveals a nuclear localization signal similar to that of SV40 large T antigen.

Introduction

The v-*abl* oncogene, the transforming gene of Abelson murine leukemia virus, arose from a recombination event between the Moloney murine leukemia virus genome and a normal mouse cellular gene, c-*abl* (Abelson and Rabstein, 1970; Goff et al., 1980). The product of the v-*abl* gene is a 160 kd fusion protein, of which the N-terminal 235 amino acids are encoded by the retroviral *gag* gene while the remainder of the protein is encoded by c-*abl*–derived sequences. p160$^{gag/v\text{-}abl}$ is N-myristoylated on the N-terminal glycine of *gag*, has intrinsic tyrosine kinase activity, and can transform fibroblasts in vitro and pre-B lymphocytes both in vitro and in vivo (Scher and Siegler, 1975; Rosenberg et al., 1975; Rosenberg and Baltimore, 1976; Witte et al., 1980; Sefton et al., 1982). The v-*abl* protein is one of a family of myristoylated cytoplasmic protein-tyrosine kinases of which the 60 kd c-*src* gene product is the prototype; however, the v-*abl* gene product is distinct from proteins of the *src* family in that it also contains an approximately 70 kd unique C-terminal domain.

The mechanism of transformation of cells by the v-*abl* protein or other members of its family is unknown. Tyrosine kinase activity is thought to be essential to the transforming ability because transformation is temperature-dependent in cells infected with an Abelson virus containing a temperature-sensitive mutation affecting tyrosine kinase activity (Rosenberg and Witte, 1988). Despite extensive searches for potential substrates of transforming protein-tyrosine kinases such as v-*abl* and v-*src* (Hunter and Cooper, 1986; Kamps et al., 1986; Bell et al., 1987), no substrates critical for transformation or for normal function have been identified, nor is it known if the same sets of proteins are required for normal and oncogenic activities. Without the definition of physiologically relevant substrates, it has been difficult to assess whether the mechanism of oncogenic activation of cytoplasmic tyrosine kinases reflects a change in enzymatic activity (for example, increased V_{max} for a normal substrate) or whether activation results from a change in a nonenzymatic parameter such as interaction with another protein or subcellular localization. While studies comparing p160$^{v\text{-}abl}$ with p150$^{c\text{-}abl}$ have suggested an increase in kinase activity coincident with oncogenic activation (Ponticelli et al., 1982), no comparison of the subcellular localization of the v-*abl* and c-*abl* proteins has been possible because of the low abundance of the normal cellular protein.

Studies of the subcellular localization of the v-*src* gene product in transformed chicken fibroblasts have shown that a significant fraction is associated with the inner surface of the plasma membrane, with particular affinity for regions of cell–cell contact and adhesion plaques (Rohrschneider, 1979, 1980; for review, see Kruegel et al., 1983), while a distinct fraction of p60$^{v\text{-}src}$ may be localized in juxtanuclear structures (Resh and Erickson, 1985). The amino terminus of the *src* protein is also myristoylated (Schultz et al., 1985), and mutants that lose the myristoylation site can no longer transform fibroblasts and are not membrane associated (Kamps et al., 1985; Buss et al., 1986), suggesting that access to some critical membrane-associated substrate may be essential for the transformation of fibroblasts by this protein. In Abelson murine leukemia virus–transformed rat fibroblasts, the v-*abl* gene product is also found at the membrane and at sites of cell–cell and cell–substratum contact (Rohrschneider and Najita, 1984).

The mouse homolog of the v-*abl* gene is denoted c-*abl*, and is transcribed into two major mRNA transcripts which differ at their 5′ ends. These two transcripts arise from splicing of two distinct 5′ exons, which are transcribed from separate promoters, to a set of common exons in the c-*abl* gene (Ben-Neriah et al., 1986). The two transcripts produce two distinct products, types I and IV c-*abl* proteins (referred to as c-*abl* (I) and (IV), respectively), which differ only at their N-termini. The c-*abl* (IV) protein is myristoylated on the N-terminal glycine (Jackson and Baltimore, 1989), while the c-*abl* (I) protein is predicted from its sequence not to be myristoylated. Simple overexpression of either type I or type IV protein via a retroviral expression system is insufficient for transformation of NIH 3T3 cells, but a deletion of 53 amino acids from an N-terminal region

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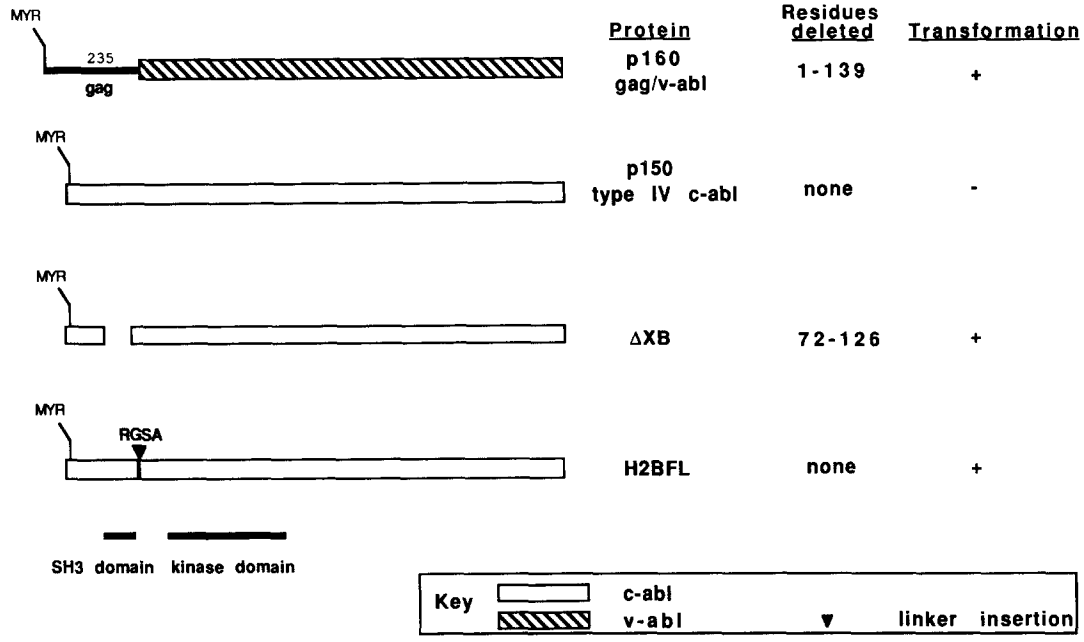

Figure 1. Structure and Transforming Properties of *abl* Proteins

Details of construction of the mutant ΔXB are given in Jackson and Baltimore (1989); construction of the linker insertion mutant H2BFL is given in Experimental Procedures. Numbering is in amino acid residues of the native c-*abl* (IV) protein. The SH3 domain is defined in the text (see Discussion).

of the c-*abl* (IV) protein is sufficient to activate its transforming potential fully, both with respect to fibroblast and B lymphoid cell transformation in vitro, and leukemogenicity in vivo (Jackson and Baltimore, 1989; see Figure 1). Conservation of this domain among cytoplasmic tyrosine kinases, phospholipase C-II, and the v-*crk* transforming protein suggests that it is a regulatory domain (Stahl et al., 1988; Mayer et al., 1988; for review, see Pawson, 1989).

We used indirect immunofluorescence to examine the subcellular localization of transforming and nontransforming Abelson protein variants expressed in NIH 3T3 cells. We found that the overexpressed c-*abl* (IV) protein is largely nuclear in location. Using a transient expression/immunofluorescence assay system in COS-1 cells, we mapped a nuclear localization signal in the type IV protein to the *abl*-specific region C-terminal to the tyrosine kinase domain. Deletion of the 53 amino acid N-terminal putative regulatory region of the c-*abl* (IV) protein results in the localization of the protein to the cytoplasm, along with activation of its transforming potential.

Results

Immunofluorescence Shows the c-*abl* (IV) Protein Is Nuclear

The amount of endogenous c-*abl* protein in NIH 3T3 cells is insufficient for immunofluorescence to be observed (R. Van Etten, unpublished data). The mouse c-*abl* (IV) protein has been overexpressed approximately 10-fold in the cell line BETA, a derivative of NIH 3T3 cells stably transfected with a retroviral expression construct containing a full-length cDNA of c-*abl* (IV) (Jackson and Baltimore, 1989). Indirect immunofluorescence of BETA cells was performed using two different immune reagents directed against the C-terminus of the *abl* protein: affinity-purified rabbit polyclonal antisera (directed against pEX5, a bacterial fusion protein containing the C-terminus of *abl* (Konopka et al., 1984; see Experimental Procedures) or purified monoclonal antibody 24-21 (also directed against pEX5; Schiff-Maker et al., 1986). A large fraction of the protein was found to be in the nucleus, the rest was localized diffusely throughout the cytoplasm at a low concentration, or concentrated at the plasma membrane (Figures 2A, 2B, and 2E). The specificity of the staining was shown by loss of immunofluorescence when the primary antibody was blocked by preincubation with purified C-terminal domain (pEX5) protein (Figures 2C and 2F), or when purified preimmune rabbit IgG at the same concentration was used as primary antibody (Figure 2D). Omission of primary antibody resulted in no detectable fluorescence (data not shown).

Examination of cells by confocal laser scanning microscopy (CLSM) allows one to section a cell optically and thereby eliminate contributions to fluorescence from structures outside the plane of focus (Brakenhoff et al., 1985). This feature can reveal structural details that are not apparent with conventional fluorescence microscopy. In Figure 3A, a BETA cell stained with anti-C-terminal (pEX5) sera was scanned through a mid-nuclear level. The staining of cytoplasmic filamentous structures that are oriented longitudinally in the cell, reminiscent of F-actin "stress fibers," is readily apparent. Double-labeling experiments with FITC-conjugated phalloidin, which preferentially stains F-actin, have confirmed that these structures are actin filaments (R. Van Etten, unpublished data). High power CLSM of the BETA cells showed that the nu-

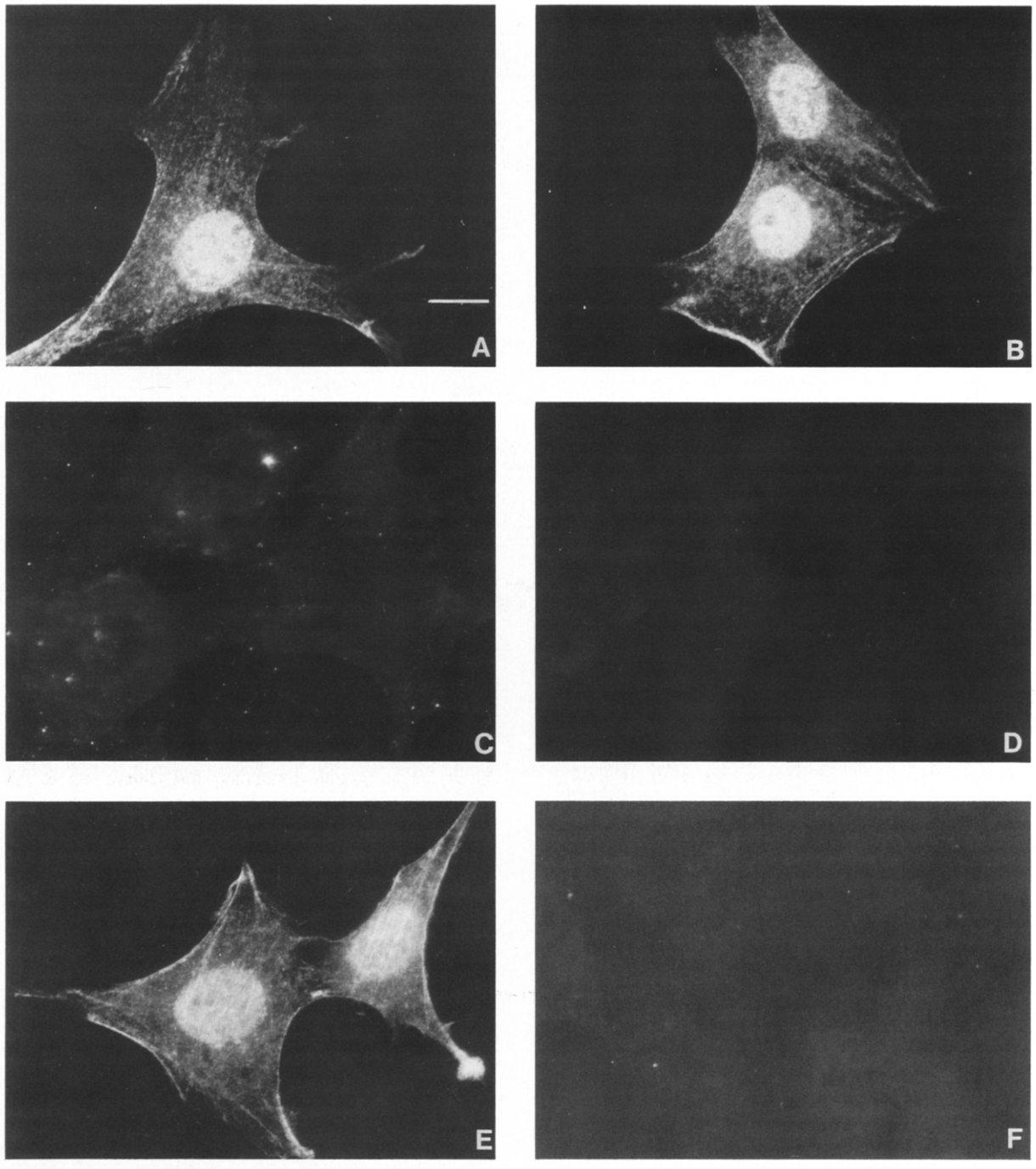

Figure 2. Indirect Immunofluorescence of Cells Overexpressing $p150^{c\text{-}abl\ (IV)}$ Demonstrate the Nuclear Localization of the Protein

Cells were fixed and permeablilized in methanol and acetone. Primary antibody was as follows. (A) and (B): affinity-purified anti-C terminal sera (pEX5). (C): anti-pEX5 adsorbed with pEX5 fusion protein. (D): preimmune rabbit IgG. (E): monoclonal antibody 24-21 (directed against *abl* C-terminus [pEX5]). (F): monoclonal antibody 24-21 adsorbed with pEX5 fusion protein. Secondary antibody was rhodamine-conjugated donkey anti-rabbit IgG in (A), (B), (C), and (D), and rhodamine-conjugated goat anti-mouse IgG in (E) and (F). Bar = 10 μm.

clear staining was relatively homogeneous throughout the nuclear matrix, sparing the nucleoli, but may exhibit a small amount of increased concentration at the nuclear envelope (Figure 3B). Quantitation of the fluorescence image obtained from CLSM indicated that between 40% to 60% of the type IV protein in the cell was nuclear (see Experimental Procedures).

Transforming *abl* Proteins Are Cytoplasmic or Plasma Membrane Associated

N54 cells are NIH 3T3 fibroblasts that are productively infected with Abelson murine leukemia virus and which express large amounts of the $p160^{gag/v\text{-}abl}$ protein. Indirect immunofluorescence of these cells showed the cytoplasm stained diffusely at a relatively high level, with little or no

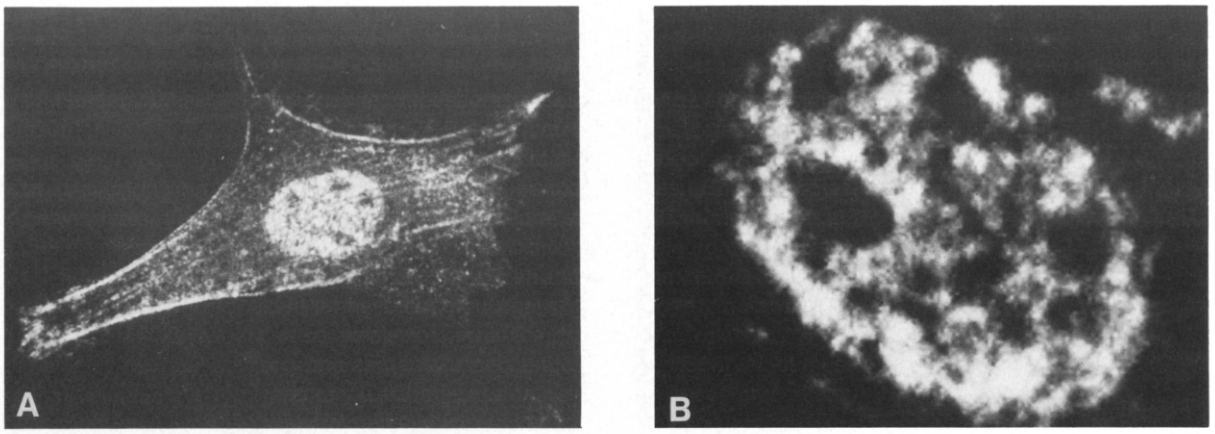

Figure 3. Confocal Laser Scanning Microscopy of BETA Cells Demonstrates That p150$^{c\text{-}abl\ (IV)}$ Is Localized throughout the Nucleus

Cells were fixed and permeabilized with methanol and acetone and stained with affinity-purified anti-pEX5 antibody followed by rhodamine-conjugated donkey anti-rabbit IgG. Scanning and data collection on a Bio-Rad MRC-500 confocal microscope was performed through a mid-nuclear level of the cell. (A) Magnification, 1300×. (B) Magnification, 5100×, showing nuclear detail.

nuclear staining; preimmune sera gave no staining (Figures 4A and 4B). The v-*abl* protein showed particularly high concentration at the plasma membrane and regions of cell–cell contact, in agreement with previous reports (Rohrschneider and Najita, 1984). The cells were not stained if exposed to immune reagents without being permeabilized first, indicating that the v-*abl* protein was associated with the inner surface of the plasma membrane (data not shown). More detailed studies showed preferential association of some of the v-*abl* protein with adhesion plaques and the Golgi apparatus (R. Van Etten, unpublished data).

Indirect immunofluorescence of fibroblasts expressing ΔXB, the activating deletion mutant of c-*abl* (IV) (see Figure 1), showed a pattern essentially identical to that seen with v-*abl* (Figure 4C), with the majority of the protein distributed throughout the cytoplasm, membrane, and regions of cell–cell contact, and no appreciable amount in the nucleus. Thus, a deletion of 53 amino acids, which serves to activate the transforming potential of the c-*abl* (IV) protein, is accompanied by a dramatic change in subcellular localization.

A common feature of p160$^{gag/v\text{-}abl}$ and ΔXB is that each lacks N-terminal sequences of the c-*abl* (IV) protein that could be important in directing the localization of the protein. We examined the localization of an activated transforming mutant of c-*abl*, H2BFL, which differs from the wild-type protein by only a linker insertion mutation near the C-terminal border of the region deleted in the ΔXB protein, giving an insertion of four additional amino acids (see Figure 1). This mutant protein was also localized to the cytoplasm (Figure 4E), which suggests that cytoplasmic localization is an intrinsic property of activated forms of *abl*, and not simply the result of removing N-terminal sequences.

Transient Expression of Transforming and Nontransforming *abl* Proteins in COS-1 Cells Reflects Their Localization in Stable NIH 3T3 Cell Lines

To study the requirements for nuclear localization, we first investigated whether a transient expression/immunofluorescence system using replicating SV40-based vectors would reproduce the localization seen in fibroblast cell lines stably expressing the c-*abl* (IV) and ΔXB mutant proteins. The c-*abl* (IV) and ΔXB cDNAs were cloned into the COS cell expression vector PJ3Ω (see Experimental Procedures), a replicating vector that directs expression under the control of the SV40 early region promoter, and were introduced into COS-1 cells by DEAE-dextran transfection. The cells were assayed by indirect immunofluorescence 48–60 hr after transfection. The majority of the c-*abl* (IV) protein was found in the nucleus, again sparing the nucleoli, with a minimum of cytoplasmic staining and some increased concentration at the plasma membrane (Figure 5A). Mock-transfected cells showed a very low level of background fluorescence (data not shown).

In contrast, the ΔXB protein was excluded from the nucleus (Figure 5B). Many of the cells showed an increased concentration of ΔXB protein in the region of the cytoplasm closely associated with the nucleus; this "juxtanuclear" staining was not likely to be the result of "leakage" of the ΔXB protein out of the nucleus due to damage to the nucleus during fixation or staining because the fine structure of the nucleus appeared intact under phase microscopy, and the nucleus appeared intact after staining with the DNA-specific fluorochrome H22358 (data not shown). In addition to this "juxtanuclear" staining, the ΔXB protein was localized diffusely throughout the cytoplasm, with some apparent increased concentration at the cell membrane. In 10% to 20% of cells expressing the protein,

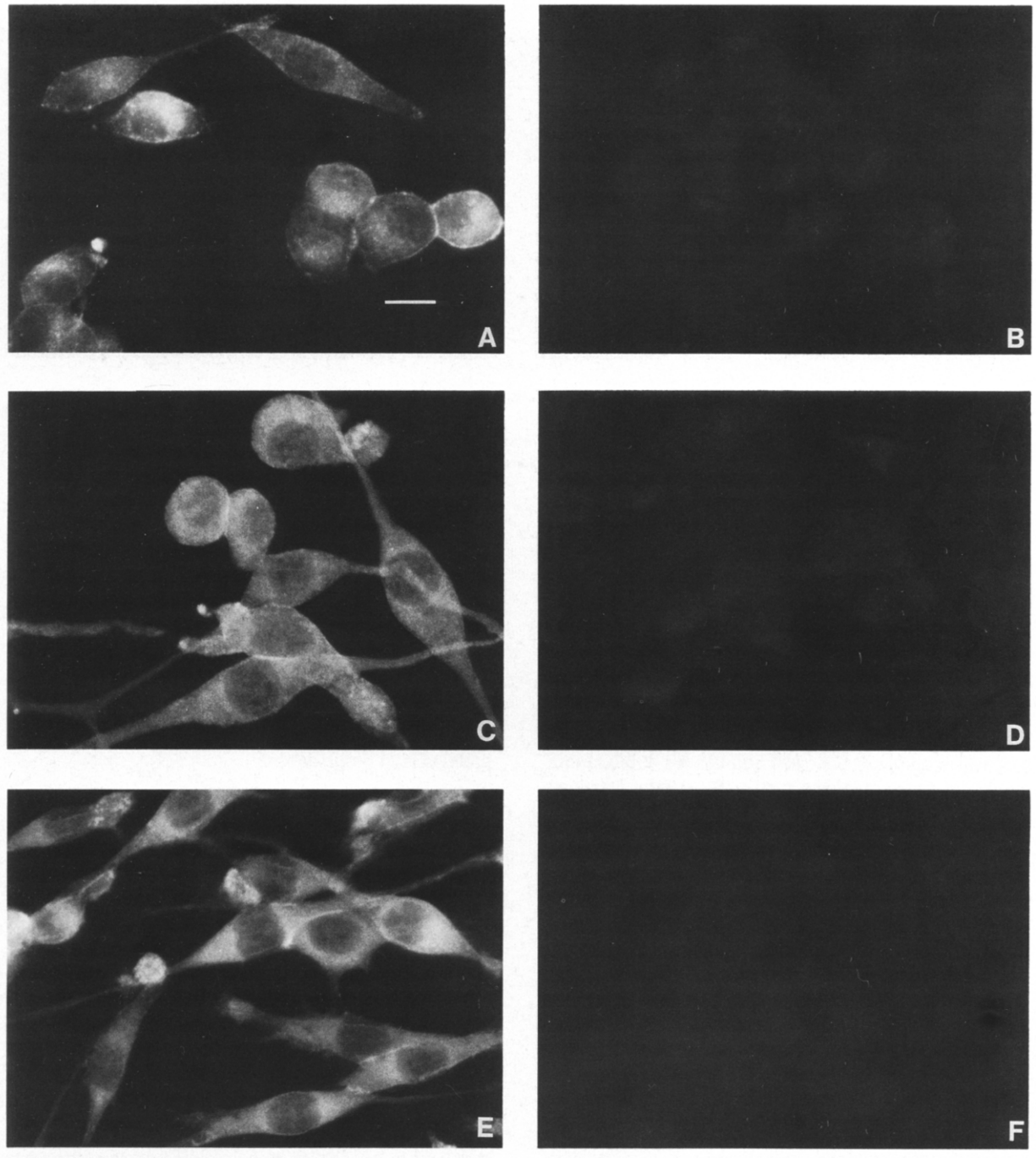

Figure 4. Indirect Immunofluorescence of Transforming *abl* Proteins in NIH 3T3 Fibroblasts Shows Cytoplasmic and Plasma Membrane Association

(A) and (B): $p160^{gag/v\text{-}abl}$ in cells fixed and permeablilzed with methanol and acetone. Primary antibody in (A) was a 1:1 mixture of anti-pEX4 and anti-pEX5 sera (both directed against the *abl* C-terminus) at a 1:300 dilution. In (B) it was rabbit preimmune serum at a 1:300 dilution. (C) and (D): deletion mutant ΔXB in cells fixed with paraformaldehyde and permeabilized with Triton X-100. Primary antibody in (C) was a 1:1 mixture of anti-pEX4 and anti-pEX5 sera at a 1:200 dilution. In (D) it was rabbit preimmune serum at a 1:200 dilution. (E) and (F): linker insertion mutant H2BFL in cells fixed and permeabilized with methanol and acetone. Primary antibody in (E) was monoclonal antibody 24-21 (directed against the *abl* C-terminus (pEX5)). In (F) it was monoclonal antibody 24-21 adsorbed with pEX5 fusion protein. Secondary antibody was rhodamine-conjugated donkey anti-rabbit IgG in (A), (B), (C), and (D), and rhodamine-conjugated goat anti-mouse IgG in (E) and (F). Bar = 10 μm.

the ΔXB protein was also found in the nucleus (data not shown); the significance of this finding is unknown, but may reflect the ability of the ΔXB protein to enter the nucleus in COS cells under certain circumstances. Such nuclear staining was never found in any ΔXB-transformed NIH 3T3 fibroblasts. Thus, in most cells, the immunofluo-

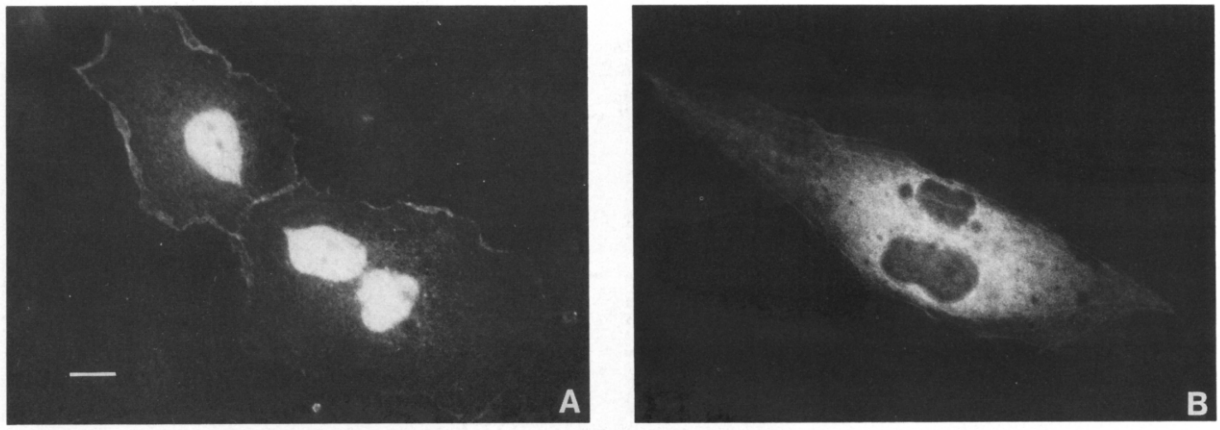

Figure 5. Indirect Immunofluorescence of COS-1 Cells Transiently Expressing *abl* Proteins Recapitulates the Localization Pattern Seen in Stable Lines

Cells were fixed and permeablilzed with methanol and acetone 48–60 hr after transfection. Primary antibody was a 1:1 mixture of crude anti-pEX4 and anti-pEX5 sera (both directed against the *abl* C-terminus) at a 1:100 dilution; secondary antibody was rhodamine-conjugated donkey anti-rabbit IgG. (A) c-*abl* (IV). (B) ΔXB. Bar = 10 μm.

rescence pattern of the *abl* proteins transiently expressed in COS cells faithfully duplicates the pattern seen in stably expressing NIH 3T3 fibroblast cell lines.

A Nuclear Localization Signal Similar to the SV40 Large T Signal Mapped to the C-Terminal Domain of the c-*abl* (IV) Protein

Proteins larger than 50–60 kd are too large to enter nuclei by passive diffusion (Dingwall and Laskey, 1986), and a number of nuclear proteins such as SV40 large T antigen contain specific sequences which function to direct nuclear localization (Kalderon et al., 1984; Richardson et al., 1986; Wychowski et al., 1986; Lyons et al., 1987; Bürglin and De Robertis, 1987; Dang and Lee, 1988). Such sequences are typically short motifs of basic amino acids. Inspection of the c-*abl* (IV) amino acid sequence revealed a motif of five contiguous lysines between residues 624 and 628, and we constructed a series of truncation mutants to determine if this motif was required for nuclear localization (Figure 6A). A series of 3′ truncation mutants of the c-*abl* (IV) cDNA were generated, and the subcellular localization of the corresponding C-terminal truncated c-*abl* (IV) proteins was determined in the COS cell immunofluorescence assay system. Western blotting demonstrated that the mutant proteins were expressed to high levels in the COS cells and had the expected sizes (P. Jackson, unpublished data).

While the truncated proteins encoded by mutants ΔXho and ΔNar retained nuclear localization (Figures 6B and 6C), the protein product of mutant ΔBcl showed a dramatically different localization, with little or no protein in the nucleus, and the majority of the protein located diffusely throughout the cytoplasm (Figure 6D). The sequence between the Bcl I and Nar I sites encodes 7 amino acids (Lee et al., 1985), most strikingly the pentalysine motif (Figure 6A), which is similar to nuclear localization signals found in several viral and cellular proteins (Table 1). A site-directed mutant replacing the five consecutive lysine residues of the full-length c-*abl* (IV) protein with five glutamines, Q5, was constructed and assayed in the COS cell system. This mutant was also deficient in nuclear localization (Figure 6E), demonstrating that the pentalysine motif is specifically required for nuclear localization. Confirmation of the localization of the C-terminal mutants of the c-*abl* (IV) protein in NIH 3T3 cells stably expressing these proteins is in progress.

Discussion

We have shown that a large fraction of the c-*abl* (IV) protein is nuclear when overexpressed in NIH 3T3 cells. Nuclear localization is also seen when the type IV protein is expressed in cells of a different species (COS-1 cells) and is dependent on the presence of a canonical nuclear localization signal, which has been mapped to a region unique to *abl*, C-terminal of the tyrosine kinase domain. The nuclear localization signal (KKKKK) is similar to other such signal sequences previously identified in a variety of viral and cellular proteins. Activation of transforming ability of the type IV protein by a deletion or small insertion in an N-terminal regulatory region is also associated with a change in localization of the protein from the nucleus to the cytoplasm. These results raise two distinct issues: the normal role of the c-*abl* protein and the mechanism of activation of transforming ability of the *abl* proteins.

Before discussing the significance of these observations, it is important to explore their limitations. First, detection of nontransforming *abl* proteins by immunofluorescence requires them to be overexpressed by at least 10-fold. Thus, it might be that the nuclear accumulation of c-*abl*(IV) is an artifact. This is unlikely because the overexpression is not extreme, the protein is too large for passive transport into the nucleus and thus is likely to require a signal for transport, and, most convincingly, has a well-

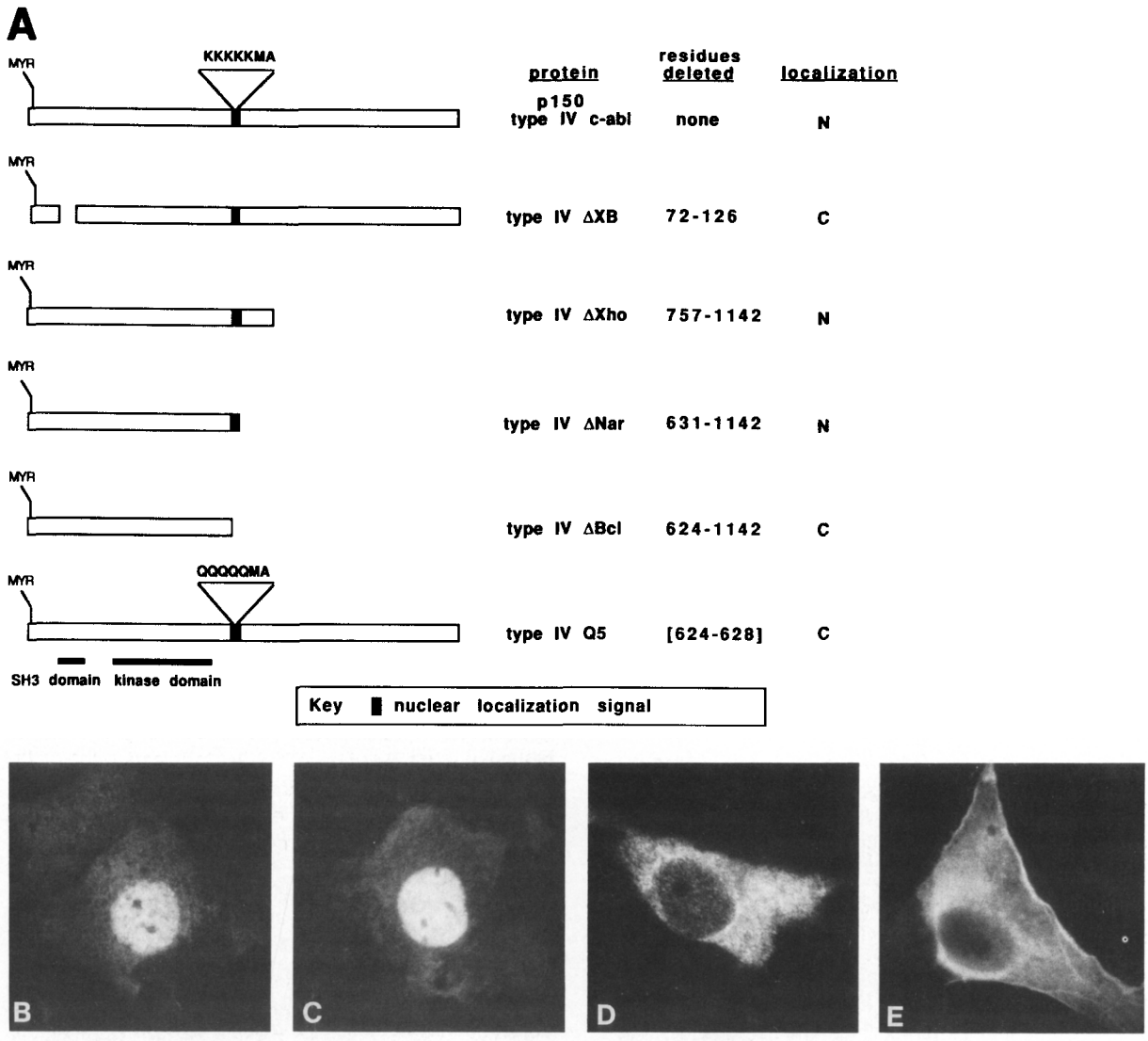

Figure 6. Mapping of a Nuclear Localization Signal in the C-Terminal Domain of the c-*abl* (IV) Protein Similar to the Signal in SV40 Large T by Indirect Immunofluorescence in COS-1 Cells

(A) Map of the type IV c-*abl* mutants ΔXho, ΔNar, ΔBcl, and Q5. Positions indicated are amino acid residues. The amino acid sequence between the NarI and BclI sites is indicated. For mutant Q5, the indicated amino acids were mutated from lysine to glutamine. Details of construction of the mutants are given in Experimental Procedures. (B)–(E): Indirect immunofluorescence of of COS-1 cells transfected with the indicated c-*abl* (IV) mutant. (B): ΔXho. (C): ΔNar. (D): ΔBcl. (E): Q5. Primary antibody for (B), (C), and (D) was monoclonal antibody 19-84 (directed against the *abl* tyrosine kinase domain); primary antibody for (E) was monoclonal antibody 24-21 (directed against the *abl* C-terminus). Secondary antibody was rhodamine-conjugated goat anti-mouse IgG.

defined nuclear localization signal similar to known signals. Because the transforming variants of the *abl* proteins are, in general, expressed at a level two to five times that seen in the cells expressing the highest levels of c-*abl* (IV) protein (Jackson and Baltimore, 1989), a second possibility is that the apparent changed distribution of the transforming ΔXB mutant might be artifactual. The increased level of expression might have overwhelmed the capacity of the nucleus for the protein, with the majority of the protein cytoplasmic as a result. However, this interpretation is unlikely for two reasons. Quantitation of the absolute amount of fluorescence in the nucleus of ΔXB cells by confocal laser scanning microscopy indicates that it is severalfold lower than the amount present in the nucleus of the c-*abl* (IV) overexpressers, suggesting that "saturation" of the nucleus with *abl* protein is not the reason for the predominantly cytoplasmic localization of the ΔXB protein. More importantly, the ΔXB protein behaves in a qualitatively different manner than c-*abl* (IV) in the COS cell assay, where both proteins are expressed to about the same level (data not shown). It should be emphasized, however, that the normal c-*abl* (IV) protein is not wholly nuclear: our quantitation shows about half the protein to be nuclear and half to be at various places in the cytoplasm. Two very important unanswered questions are whether a given c-*abl* (IV) molecule can translocate between nu-

Table 1. Comparison of the Nuclear Localization Sequence of p150$^{c\text{-}abl\ (IV)}$ with those of Several Viral and Cellular Proteins

Protein	Sequence	Reference
SV40 large T	PKKKRKV	Kalderon et al. (1984)
SV40 VP1	APTKRKGSC	Wychowski et al. (1986)
Polyoma large T	PKKARED	Richardson et al. (1986)
Adenovirus E1A	KRPRP	Lyons et al. (1987)
X. laevis nucleoplasmin	GQAKKKKLD	Bürglin and De Robertis (1987)
Human c-*myc*	PAAKRVKLD	Dang and Lee (1988)
Murine c-*abl* (IV)	SALIKKKKKMAP	

The single amino acid code is utilized. Basic amino acid residues are underlined.

cleus and cytoplasm, and whether there is any difference between the nuclear and cytoplasmic forms of the protein, either in covalent structure or in interactions with other proteins. For example, the c-*abl* (IV) protein is predicted from its sequence to be myristoylated on the N-terminal glycine residue, and the protein is labeled in vivo with [^{3}H]myristic acid with the same efficiency as the viral protein p160$^{gag/v\text{-}abl}$, but the absolute fraction of either protein that is myristoylated is unknown. Therefore, it is not known whether any of the nuclear c-*abl* (IV) protein is myristoylated.

The presence of the c-*abl* (IV) protein in the nucleus makes it the first known nuclear protein-tyrosine kinase. The insulin receptor has been reported to be translocated to the nucleus after insulin binding, but no visualization of the proteins has been reported (Podlecki et al., 1987). The presence of the c-*abl* (IV) protein in the nucleus presumably reflects part of its role in signal transduction. Although several transmembrane protein-tyrosine kinases are known receptors for polypeptide growth factors, and their effect on nuclear events associated with growth has been documented, no direct link to the nucleus for these receptors or any of the cytoplasmic tyrosine kinases has been observed. There are several candidates for possible nuclear substrates for the c-*abl* (IV) tyrosine kinase. Foulkes and co-workers found a small fraction of the v-*abl* protein to be nuclear in transformed murine fibroblasts, and they purified a distinct set of phosphotyrosine-containing nuclear proteins in v-*abl* transformed cells, several of which preferentially bound to mouse DNA over E. coli DNA (Bell et al., 1987). Although our data do not confirm the presence of nuclear v-*abl*, it is possible that some of these proteins may be targets for the c-*abl* (IV) kinase. The mammalian homolog of the fission yeast *cdc2* gene product (p34) has been shown to be phosphorylated on tyrosine, and loss of phosphotyrosine is associated with assembly of p34 into a macromolecular complex and activation of intrinsic serine kinase activity, both of which are thought to be necessary for the G2 to M transition in the cell cycle (Draetta and Beach, 1988). In this regard, it is interesting that the NIH 3T3 cells overexpressing the c-*abl* (IV) protein grow significantly more slowly than the parental 3T3 cells by a factor of 2.5 to 3.0 (Jackson and Baltimore, 1989; P. Jackson, unpublished data). The murine *cdc2* gene product is therefore another potential substrate for the type IV kinase. In this model, the predicted effect of overexpression of the type IV kinase would be hyperphosphorylation on tyrosine of the p34 protein, leading to slowing of growth.

The activating deletion in ΔXB as well as the four amino acid insertion in H2BFL act to cause the c-*abl* (IV) protein to be largely cytoplasmic rather than nuclear. These mutations thus abrogate the function of the nuclear localization signal, which remains intact in these proteins. While the respective roles of the ΔXB domain and the five lysines in determining nuclear localization and controlling transformation require additional analysis, two considerations suggest that they have independent functions. First, the specific region deleted from c-*abl* (IV) in the ΔXB mutant is a partially conserved domain related to the N-terminus of the c-*src* protein (denoted SH3 for *src* homology region 3; Figure 1; Jackson and Baltimore, 1989), and amino acid substitutions and deletions in the SH3 region of p60$^{c\text{-}src}$ can activate transforming activity (Kato et al., 1986; Potts et al., 1988). The SH3 region thus appears to be a negative regulatory domain within these proto-oncogene products, and mutations that abrogate the negative influence of this domain activate the inherent transforming ability of c-*abl* (IV) and c-*src*. Second, the ΔXB domain lacks any recognizable nuclear localization signal. Consistent with this is the behavior of the transforming mutant H2BFL, which has retained the SH3 domain but is cytoplasmically localized. This suggests that relocalization of these proteins is a direct consequence of mutations in SH3 that activate transforming ability, and is not due to disruption of a nuclear localization signal. Confirmation of the distinct functions of the ΔXB region and the pentalysine motif will come from an assessment of their ability to direct nuclear localization when fused to a heterologous reporter protein. It must also be determined whether mutations in the pentalysine motif do not simply activate the transforming ability of c-*abl* (IV), with relocalization as a consequence. Preliminary results indicate that neither the ΔBcl or the Q5 mutant of c-*abl* (IV) are able to transform NIH 3T3 fibroblasts (P. Jackson, unpublished data). The c-*abl* (IV) protein remains nuclear in BETA cells that have been transformed with an activated v-Ha-*ras* gene, indicating that cytoplasmic localization is an intrinsic property of activated forms of *abl* protein, and is not simply a general consequence of the transformed state (unpublished data).

Experimental Procedures

Cells and Cell Culture

The murine NIH 3T3 fibroblast lines BETA, N54, and ΔXB, stably expressing the *abl* proteins c-*abl* (IV), *gag/v-abl*, and ΔXB, respectively, were constructed, characterized, and propagated as described (Jackson and Baltimore, 1989). COS-1 cells were routinely maintained in Dulbecco's modified Eagle's medium with 10% inactivated fetal calf serum in a 10% CO_2 incubator at 37°C.

Immune Reagents

Rabbit polyclonal antisera against the v-*abl* trpE bacterial fusion proteins pEX2, pEX4, and pEX5 were produced as described (Konopka et al., 1984) and were a kind gift from Dr. Owen Witte (Dept. of Microbiology, UCLA). Hybridoma cells and tissue culture supernatants of the monoclonal antibodies 19-84 and 24-21, directed against the pEX2 and pEX5 antigens, respectively (Schiff-Maker et al., 1986), were a generous gift from Dr. Naomi Rosenberg (Tufts University School of Medicine). Rhodamine-conjugated donkey anti-rabbit or goat anti-mouse IgG were obtained from Jackson Immunoresearch, Inc. Soluble pEX5 protein for affinity purification of antisera and blocking purposes was obtained by expressing the pEX5 sequence as part of a bacterial fusion protein with the S. japonicum glutathione S-transferase protein in the Glutagene expression system (Smith and Johnson, 1988; Amrad Corporation, Victoria, Australia). Briefly, a 1.1 kb SmaI–BamHI fragment of the c-*abl* cDNA was cloned into the BamHI site of the pGEX-1 expression vector, and the correct translational reading frame confirmed by DNA sequencing. The resulting 40 kd fusion protein was purified from lysates of E. coli by affinity chromatography on glutathione-agarose (Sigma), and was shown to contain pEX5 determinants by Western blotting with anti-pEX5 antisera or monoclonal antibody 24-21 (data not shown). For affinity purification of anti-pEX5 antisera, approximately 2 mg of pEX5 fusion protein was coupled to Affigel-10 agarose beads (Bio-Rad), adsorbed with anti-pEX5 serum at 4°C for 12 hr, IgG not bound was washed off with phosphate-buffered saline (PBS), and IgG specifically bound to the column was eluted with 0.1 M glycine–HCl (pH 2.8). The eluate was immediately neutralized, pooled, and concentrated by centrifugation (Centricon, Amicon Corp.) Protein concentration was determined by Bio-Rad protein reagent, and purified antibody stored at 4°C. IgG was purified from preimmune serum by affinity chromatography on protein A–sepharose (Pharmacia) in a similar manner. Monoclonal antibody 24-21 was produced in tissue culture supernatants by growing hybridoma cells in DMEM containing 15% IFS until a stationary phase was reached. Cells were removed by centrifugation at 5000 × g for 10 min, and the monoclonal antibody purified by affinity chromatography on a goat anti-mouse IgG/sepharose column (Cappell) as described above.

Immunofluorescence

Cells were typically plated onto sterilized glass cover slips the night before at a density of approximately 1–2 × 10^4 cells/cm^2. For some experiments, cover slips were treated with purified fibronectin at 20 μg/ml (a gift from Dr. R. Hynes, Dept. of Biology, MIT) or poly-L-lysine at 1 mg/ml (Sigma) for 1 hr prior to addition of medium and cells. Cover slips were rinsed in PBS and fixed by either absolute methanol for 5 min followed by acetone for 2 min, both at −20°C, or by 3% paraformaldehyde in PBS for 20 min at room temperature. Paraformaldehyde-fixed cells were quenched in 50 mM glycine (pH 8.0) for 10 min, then permeabilized by 0.5% Triton X-100 in PBS for 5 min at room temperature. All subsequent steps were carried out at room temperature. Cells were then blocked by 5% normal donkey serum or normal goat serum (Jackson Immunoresearch) for 30 min for experiments utilizing donkey or goat secondary antibodies, respectively. Primary antibody was used at a dilution of 1:100 to 1:300 for crude rabbit antisera, at 1–10 μg/ml for affinity-purified antisera or monoclonal antibody 24-21, and as undiluted tissue culture supernatant in the case of monoclonal antibody 19-84. Where indicated, affinity-purified anti-pEX5 antisera or monoclonal antibody 24-21 were blocked with pEX5 fusion protein by incubation with a 10-fold molar excess of purified fusion protein for 30 min prior to application to cells. Incubation of primary antibody was for 45 min, followed by extensive washing in PBS. The appropriate secondary antibody was used at a 1:100 dilution for 30 min, after which the cells were washed briefly with PBS and mounted with Fluoromount anti-quenching agent (Fischer Scientific). Conventional epifluorescence microscopy was performed with a Zeiss fluorescence microscope, and confocal laser scanning microscopy performed on an MRC-500 system (Bio-Rad Corp.) Quantitation of fluorescence intensity was done with the AREA software package option. Photography of fluorescence specimens was accomplished with Kodak Tmax ASA 400 black-and-white 35 mm film, while photography of the confocal microscope screen utilized Kodak Panatomic-X ASA 32 black-and-white 35 mm film.

COS-1 Cell Transfection

Between 0.5 and 1.0 μg of CsCl-purified plasmid DNA was used to transfect monolayers of COS-1 cells at a density of 3 × 10^4 cells/cm^2 growing on sterilized glass cover slips. Transfection was by a DEAE-dextran protocol with 0.1 mM chloroquine treatment (Sompayrac and Danna, 1981; Luthman and Magnusson, 1983). After 48–60 hr, medium was removed and the cells fixed with methanol and acetone, followed by indirect immunofluorescence as described above.

Construction of C-Terminal Truncation Mutants of c-*abl* (IV)

The cDNA for c-*abl* type IV was constructed in vector pJ3Ω, provided by Jay Morgenstern (Whitehead Institute, MIT). Briefly, a 4.1 kb EcoRI to HindIII fragment from vector pPLcIV (Jackson and Baltimore, 1989) was blunted with the Klenow fragment of E. coli DNA polymerase I, fitted with BamHI linkers and ligated into the BamHI site in the vector polylinker. This plasmid pJ3cIV was used for all subsequent constructions. Mutant pJ3cIVΔNar was constructed by cutting pJ3cIV at the unique NarI site in c-*abl* and at a ClaI site in the vector polylinker 3′ to the gene; these sites were blunted with Klenow fragment, and fitted with a SpeI 14-mer linker with termination codons in all three translation frames. Mutant pJ3cIVΔBcl was constructed by growing pJ3cIV in E. coli strain GM48, cutting the demethylated DNA with BclI, blunting with Klenow, adding SpeI 14-mers, and religating. The mutant pJ3cIVQ5 was constructed by synthesizing both strands of a fragment spanning the BclI and NarI sites (changing each of the lysine codons to glutamine codons), and ligating the annealed oligonucleotides directly into the pJ3cIV cut with BclI and NarI. Mutants were re-screened using the mutant oligonucleotides and verified by sequencing.

Construction of Linker Insertion Mutant H2BFL

Mutant pPLH2B was generated by opening vector pPLcIV (a retroviral expression construct of c-*abl* [IV]; Jackson and Baltimore, 1989) at the 5′ HincII site and adding a BamHI 12-mer synthetic oligonucleotide linker (New England Biolabs) with the sequence pCGC GGA TCC GCG-OH, thus inserting the tetrapeptide RGSA between residues V138 and N139.

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Note Added in Proof

Although as reported here the Q5 mutant is cytoplasmic after transient expression in COS cells, recent evidence indicates that when stably expressed in 3T3 cells, it is partially nuclear. Apparently in some circumstances, more than one nuclear localization signal can be functional.