The EMBO Journal vol.8 no.2 pp.449-456, 1989

N-Terminal mutations activate the leukemogenic potential of the myristoylated form of c-*abl*

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Communicated by D.Baltimore

The two major forms of the c-*abl* gene differ from their activated counterpart, the v-*abl* oncogene of the Abelson murine leukemia virus by the replacement of their N-terminal sequences with viral *gag* sequences. Overexpression of p150$^{c\text{-}abl\ type\ IV}$ in a retroviral vector similar to Abelson virus does not transform NIH 3T3 fibroblasts, even though it is expressed and myristoylated at levels comparable to pp160$^{v\text{-}abl}$. Members of a nested set of deletion mutations of the N-terminus of c-*abl* type IV in this expression system will activate *abl* to transform murine fibroblasts. The smallest of these deletions, ΔXB, efficiently transforms lymphoid cells *in vitro* and causes leukemia *in vivo* demonstrating that *gag* sequences are not necessary for *abl*-induced leukemogenesis. The ΔXB mutation defines an N-terminal regulatory domain, which shares a surprising homology with chicken oncogene v-*crk* and phospholipase C-II. Although overexpression of the myristoylated form of c-*abl* does not transform cells, it nonetheless has a profound effect on cell growth.

Key words: Abelson murine leukemia virus/oncogenes/cell transformation/phospholipaseC/B lymphoid cells

Introduction

The transforming proteins of several oncogenic retroviruses contain domains that act as cytoplasmic tyrosine kinases (Witte *et al.*, 1980; Hunter and Sefton, 1980; Bishop, 1983). Similar kinase domains are associated with transmembrane receptors for peptide growth factors (Ushiro and Cohen, 1980; Downward *et al.*, 1984; Sherr *et al.*, 1985; Yarden *et al.*, 1986). While the receptors have an apparent function—to transduce signals from the cellular exterior into the cytoplasm—the cytoplasmic kinases lack any clear mode of action. We do not know what factors regulate them, where in the cell such factors may act, or what portions of the kinase proteins are involved in regulatory interactions. While mutational analysis of the cytoplasmic kinases—the *src* family, *abl*, and *fps*—has defined the catalytic domains of the gene products, regulatory domains of the normal cellular genes have not been as well defined (for review, see Jove and Hanafusa, 1987). In part, the lack of understanding of control regions of these kinases is due to the focus on studying transformation-defective mutants of already activated oncogenes. While this type of mutation will define the functions necessary for transformation, it is not clear that such mutations suggest what normal controls are dysregulated during activation. Activation will more profitably be studied by looking at forward mutations of normal, non-transforming cellular genes. Such an approach has been used by Hanafusa and co-workers studying recovered Avian Sarcoma Viruses (rASVs), where viruses expressing c-*src* in a context similar to v-*src* in Rous Sarcoma virus are passaged until tumorigenic variants arise (Iba *et al.*, 1984; Levy *et al.*, 1986). The mutations causing activation may then be mapped, sequenced and studied biochemically. In this study, we establish a system for making forward mutations in the c-*abl* proto-oncogene by directed mutation, thereby allowing the definition of domains which negatively control the tyrosine kinase activity and transformation potential latent in the *abl* gene.

The c-*abl* proto-oncogene is the normal cellular homolog of the v-*abl* transforming gene of Abelson murine leukemia virus (A-MuLV), the etiological agent of a pre-B cell leukemia in mice (Abelson and Rabstein, 1970; Heisterkamp *et al.*, 1983; Wang *et al.*, 1984; Witte *et al.*, 1979). A-MuLV encodes a 160 kd chimeric protein containing N-terminal sequences derived from the *gag* gene of Moloney murine leukemia virus (M-MuLV) fused to c-*abl* sequence (Goff *et al.*, 1980). Biochemical studies have revealed that P160$^{gag\text{-}v\text{-}abl}$ is a protein tyrosine kinase, itself phosphorylated on tyrosine both *in vivo* and *in vitro* (Ponticelli *et al.*, 1982; Witte *et al.*, 1980). Mutational analyses with temperature sensitive and kinase defective mutants of A-MuLV indicate that the kinase activity of P160$^{v\text{-}abl}$ is essential for the transformation of fibroblasts and lymphoid cells *in vitro* (Baltimore *et al.*, 1979; Reynolds *et al.*, 1980; Rosenberg *et al.*, 1975; Rosenberg and Witte, 1980; Scher and Siegler, 1975; Witte *et al.*, 1979; Ziegler *et al.*, 1981; Takemori *et al.*, 1987).

c-*abl* protein is produced in two major forms in human and mouse cells, differing in sequence at their N-termini (Ben-Neriah *et al.*, 1986; Shtivelman *et al.*, 1986). One form, type IV, has the sequence of a protein that should be myristoylated on an N-terminal glycine and we show here that it has an attached myristoyl group. This 1142-amino acid protein migrates during electrophoresis as an ~150 kd protein and is called p150. The second major c-*abl* protein, type I, has 1122 amino acids and migrates indistinguishably from type IV, but should not be myristoylated from its sequence (Ben-Neriah *et al.*, 1986).

The genomic structure of the murine c-*abl* locus (Ben-Neriah *et al.*, 1986; Bernards *et al.*, 1987; Bernards *et al.*, 1988) and the gene products associated with it differ from the retroviral structure and v-*abl* gene product in two major ways: the expression from the endogenous control sequences versus retrovirus mediated expression and the replacement of the N-terminus of c-*abl* with the *gag* moiety from M-MuLV. To determine which of these differences was reponsible for activation, we first examined whether retroviral transduction and overexpression of the two major c-*abl* types was sufficient to transform rodent fibroblasts. Having found that overexpression of neither type IV (this

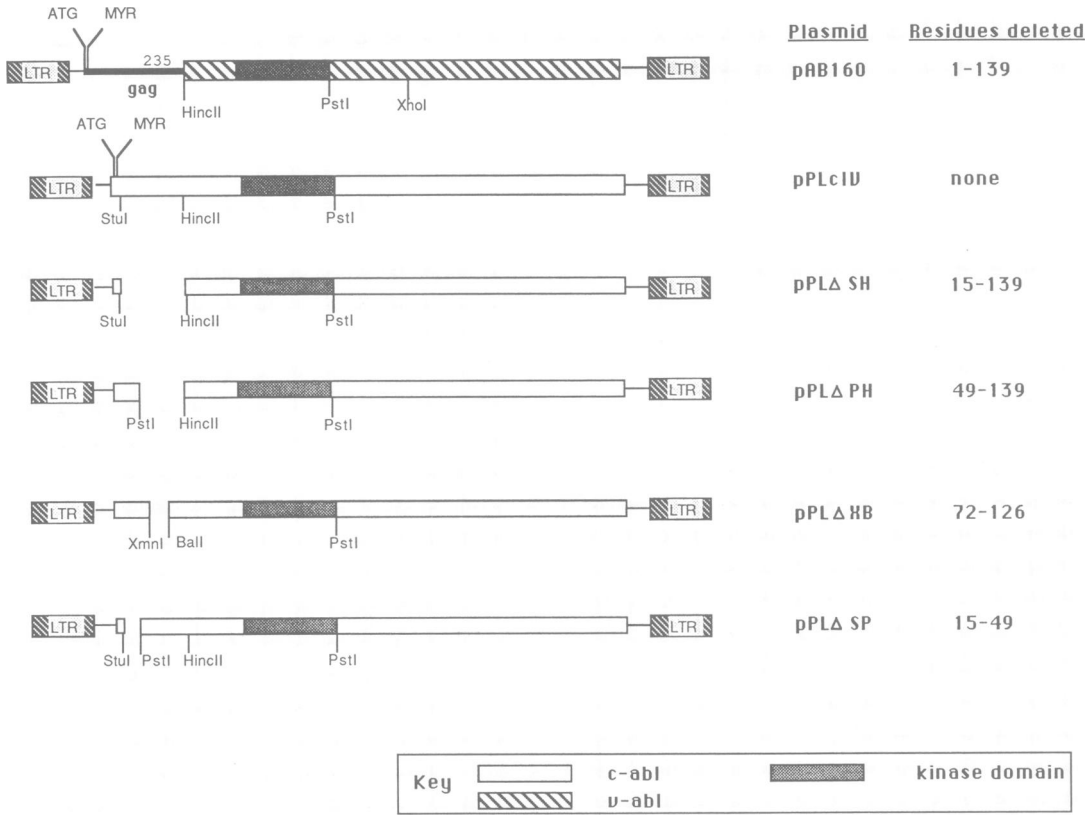

Fig. 1. Genomic arrangement of c-*abl* type IV deletion mutants. c-*abl* sequences are reconstructed cDNAs. Details of the constructions are given in Materials and methods section. The vector used is a simple retroviral vector analogous to v-*abl*: M-MuLV LTRs and mouse flanking sequences are cloned into a pUC13 backbone (Mathey-Prevot and Baltimore, 1985). Constructions are as follows: (i) pAB160, a v-*abl* proviral clone, as described (Goff *et al.*, 1981); (ii) pPLcIV, a wild-type c-*abl* type IV cDNA, with its natural ATG; (iii) pPLΔSH, a deletion of the type IV N-terminus (deleting residues 15–139) leaving only the first 14 residues of type IV; (iv) pPLΔPH, a *Spe*I linker was used to create a junction between the *Pst*I site near the beginning of the first common exon of c-*abl* and the *Hin*cII site; (v), pPLΔXB, the N-terminus of type IV was cut with *Xmn*I and *Bal*I and religated, leaving an in-frame internal deletion, and (vi) pPLΔSP, a *Xho*I linker was used to create a junction between the *Stu*I site and the *Pst*I site near the beginning of the first common exon, deleting 34 residues of type IV specific sequence.

paper), nor type I (our unpublished results) was transforming, we constructed a series of N-terminal mutations in an attempt to activate the myristoylated form of c-*abl*. We found that viruses expressing N-terminal mutants of the myristoylated form of the *abl* protein mimic many if not all of the transforming properties of A-MuLV suggesting a role for the N-terminal domain in the activation of c-*abl*, and a possible role in the normal regulation of c-*abl*.

Results

Construction of a c-abl type IV virus and mutants

A full length cDNA representing c-*abl* type IV (c-*abl* IV) was constructed in a simple retroviral vector, pPL (Mathey-Prevot and Baltimore, 1985) in a context similar to that of v-*abl* in A-MuLV. The vector provides 5′ and 3′ LTR sequences for transcriptional control and polyadenylation. This vector, pPLcIV, was used as a starting point for all subsequent mutagenesis (Figure 1).

A series of N-terminal deletion mutants was constructed (Figure 1). We chose to focus on the N-terminus because in pp160$^{v\text{-}abl}$ the first 139 residues of the predicted c-*abl* type IV gene product are removed, suggesting that deletion of this domain might be involved in activation. Prywes *et al.* (1983, 1985) had previously examined whether the *gag* sequences appended to the N-terminus of the *abl* sequences in pp160 had any positive role in transformation. Although in lymphoid cells *gag* was found to play a stabilizing role, in fibroblasts only the N-terminal 14 amino acids of *gag* were

Table I. Transformation by transfection of NIH 3T3 cells

Constructs transfected	Protein detected	Foci/μg DNA
Mock	p150[a]	<1
MoMuLV	–	<1
MoMuLV + pAB160	pp160	10^{4}[b]
MoMuLV + pPLcIV	p150	<1
MoMuLV + pPLΔSH	p135	10^{3}
MoMuLV + pPLΔPH	p140	$>10^{3}$
MoMuLV + pPLΔXB	p143	$>10^{3}$
MoMuLV + pPLΔSP	pp148	<1

[a]p150 is a tyrosine kinase immunoprecipitable with various anti-*abl* sera (for example, PEX4 or PEX5, Konopka and Witte, 1985). The species seen in kinase assays, however, may represent a mixture of c-*abl* types I and IV, a possibility supported by the expression of mRNA messages believed to represent both species in all cell lines surveyed. [b]Goff *et al.*, 1982. Although the number of foci generated by v-*abl* is ~5- to 10-fold higher than that generated by, for example, ΔXB, this need not reflect an intrinsic difference in either transforming ability nor stability of the protein product. Indeed, *gag*-related constructs have their ATG in a different context (AATATGG) than type IV related constructs (TTTATGG). According to Kozak (1986), the T in position −3 is about 5-fold less efficient than an A in the same position.

required for transformation and they were thought to provide a myristoylation site. Our mutations in c-*abl* were designed to ask first if the N-terminal 14 amino acids of c-*abl* IV, which also encode a myristoylation site from their sequence (Towler *et al.*, 1987) can provide transforming ability to the kinase function of the *abl* protein. Thus, mutant ΔSH was constructed (Figure 1), which fuses the N-terminal 14 amino acids of c-*abl* IV to the *Hin*cII site, just where *gag* is linked in v-*abl*. Since this mutant proved to be transforming (see next section), smaller deletions were constructed to localize the activating lesion. These mutations, ΔSP, ΔPH and ΔXB spanned the N-terminal sequences deleted in pp160$^{v\text{-}abl}$.

Transfection of viral constructs and isolation of overexpressing cell lines

pPLcIV and related mutants were introduced into NIH 3T3 cells in pairs of calcium phosphate transfections: one co-precipitated with a proviral clone of M-MuLV, pZAP (as described previously, Goff *et al.*, 1982), another with pSV2*neo* (Southern and Berg, 1982). Transforming variants were analyzed by isolating clonal lines from M-MuLV mediated foci. Non-transforming constructs were studied by screening clonal *neo*^{r} cell lines for high expression.

Co-transfection of pPLcIV with pZAP revealed no foci at 14 days post-transfection in multiple independent transfections (see Table I), indicating that the c-*abl* type IV cDNA does not transform NIH 3T3 cells. Multiple *neo*^{r} clones from two separate transfections were screened for functional expression of c-*abl* protein by immune complex kinase assay, and a high expresser line, BETA, and a modest expresser line, BAN2B, one from each transfection, were chosen for further study (see below). Figure 2 shows the non-transformed morphology of these type IV over-expressing clones.

Transfections of pPLΔPH and ΔXB gave rise to foci with efficiency, latency and morphology similar to a v-*abl* proviral clone, pAB160; pPLΔSH gave rise to foci 2- to 4-fold less efficiently than ΔPH or ΔXB (compare foci/μg DNA in Table I). Thus, various modifications of the N-terminus of c-*abl* IV activate the transforming ability of this cDNA.

Clonal cell lines from foci were isolated. Typically 10 lines were screened for expression by *in vitro* kinase assay and the two lines expressing the highest levels were further characterized. In each case, multiple lines representing the same mutant behaved similarly in all biological assays. pPLΔSP gave rise to no detectable foci. Thus, some N-terminal deletion mutations are incapable of activating the transforming ability of c-*abl* IV. *Neo*^{r} lines expressing this protein were screened for high expression and three ΔSP clones were chosen for further study. The morphology of cells transformed by ΔSH, ΔPH, and ΔXB is comparable to that of a v-*abl* transformed line, N54; ΔSP clones were not transformed (Figure 2).

When non-transforming clone pPLcIV was co-transfected with pZAP and foci scored at 21 days, a few foci were observed. These foci appeared with longer latency and were qualitatively different from foci generated by transforming clones. First, transforming clones produce numbers of foci proportional to the number of cells plated after transfection. The non-transforming variants do not give foci in proportion to the initial dilution of cells: some very high dilutions will give foci and some low dilutions none at all. Second, when grown into clones of producing lines, these clones give high

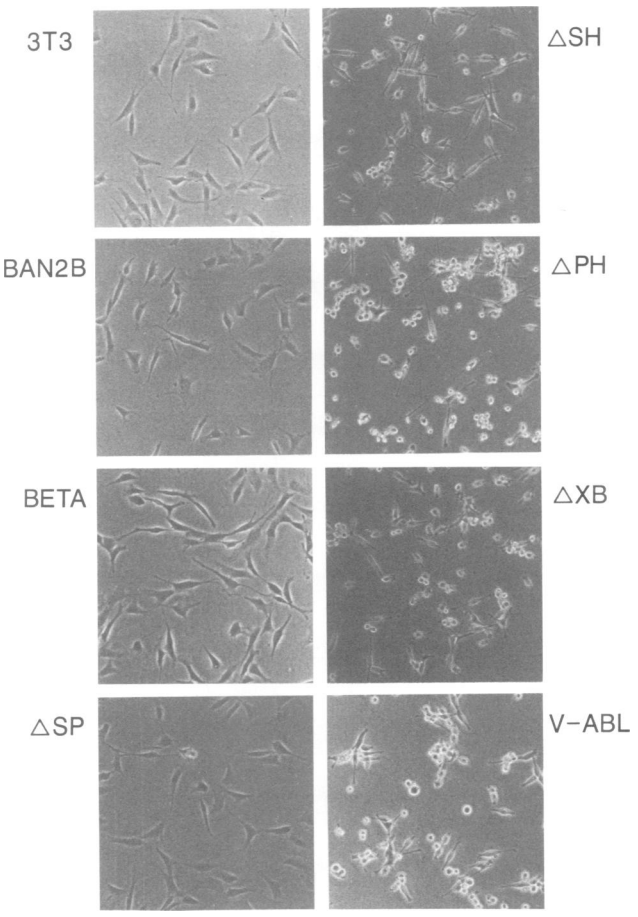

Fig. 2. Morphology of NIH 3T3 cells overexpressing c-*abl* type IV mutants.

focus-forming titers. These observations are consistent with a recombinational event generating a transforming mutant as seen in the generation of a *gag−bcr−abl* hybrid capable of transforming NIH 3T3 cells from a *bcr−abl* non-transforming virus co-transfected with pZAP (Daley *et al.*, 1987).

Expression of c-abl type IV and related mutants in NIH 3T3 cells

Expression of c-*abl* IV was tested by immunoprecipitation with antisera directed against the C-terminus of the *abl* gene product (Konopka *et al.*, 1984; Konopka and Witte, 1985). Because NIH 3T3 cells have an endogenous p150$^{c\text{-}abl}$ protein, overexpression can only be judged quantitatively. [^{35}S]methionine labeling of clone BETA (type IV overexpresser) showed clear overexpression relative to parental NIH 3T3 cells (Figure 3A: compare control in lane 2 to overexpresser in lane 4). Densitometric scanning of representative autoradiograms suggests overexpression of ~10-fold by [^{35}S]methionine labeling for clone BETA (these results have been verified by immunoblotting; Van Etten, Jackson, and Baltimore, unpublished results). Furthermore, the level of expression of p150 in this line is clearly comparable to that of related transformed lines expressing pp140$^{\Delta PH}$ (Figure 3A, lane 6) and pp143$^{\Delta XB}$ (lane 5). These transformed clones were also the highest expressers of each protein: lower levels of protein expression were sufficient for transformation by ΔXB and ΔPH mutants.

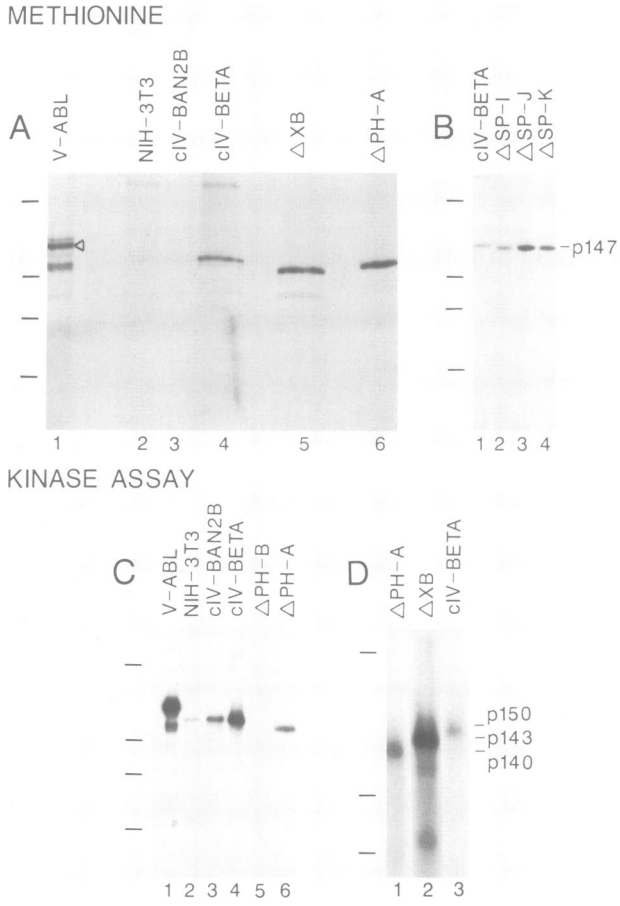

Fig. 3. Immunoprecipitation of c-*abl* type IV N-terminal mutants **A**, **B**, [^{35}S]methionine label. NIH 3T3 cells expressing the indicated mutant proteins were labeled with [^{35}S]methionine as described in Materials and methods. (**A**, **B**) All lines were immunoprecipitated with anti-*abl* sera[PEX4/PEX5]. (**A**) **Lane 1**, N54 cells (a pp160$^{v\text{-}abl}$ transformed NIH 3T3 line); **lane 2**, NIH 3T3 cells; **lane 3**, (a low expresser of c-*abl* type IV, p150); **lane 4**, BETA (a high expresser of type IV); **lane 5**, ΔXB (pp143) clone; **lane 6**, ΔPH-A (pp140) clone. (**B**) **Lane 1**, BETA (as in A, lane 5); **lanes 2**, **3**, **4**, three clones of ΔSP (p147). In **A** and **B**, immunoprecipitates were normalized for trichloroacetic acid-precipitable counts. (**C**, **D**) Immune complex kinase assays. All immunoprecipitations used the PEX4/PEX5 cocktail as above. Lysates were normalized for protein concentration. (**C**) **Lane 1**, N54 (2/5 of sample); **lane 2**, NIH 3T3 cells; **lane 3**, BAN 2B (type IV low expresser); **lane 4**, BETA (type IV high expresser); **lanes 5**, **6**, ΔPH clones. (**D**) **Lane 1**, ΔPH-A clone; **lane 2**, ΔXB clone; **lane 3**, BETA (type IV). Mol. wt markers shown to the left of each autoradiogram are 200, 116, 92.5, and 66 kd.

Because constructs representing the latter two mutants transform at frequencies at least 10^3 × higher than pPLcIV in transfection assays, it appears that retroviral transduction and 10-fold overexpression of c-*abl* type IV is not sufficient for transformation of NIH 3T3 cells. Clones of non-transforming mutant p148$^{\Delta SP}$ expressed at levels similar to expression of type IV in BETA cells (Figure 3B).

To determine functional expression, *in vitro* kinase assays were performed. The *in vitro* kinase activity of p150$^{c\text{-}abl}$ immunoprecipitated from NIH 3T3, BAN2B and BETA cells roughly paralleled the amounts of protein determined by metabolic labeling (Figure 3C: cf. Figure 3A). The level of kinase activity in BETA cells was similar to that seen in transformed clone ΔPH-A (Figure 3C, lane 4 versus lane 6), or in a clone of ΔXB (Figure 3D), suggesting that absolute level of kinase activity does not strictly determine transformation, but rather that there is a qualitative difference in the respective activities of c-*abl* IV and the Δ mutants. Furthermore, some fully transformed mutants expressed low levels of kinase activity: a clone of ΔPH (Figure 3C, lane 5) has much lower kinase activity than an equivalent clone (lane 6), but both are fully transformed. The levels of expression of kinase activity in BETA cells or clones of the Δ mutants were several fold lower than that of pp160$^{v\text{-}abl}$ in N54 cells; however, lower levels of pp160 expression than that seen in this established line will transform fibroblasts (P.Jackson, G.Daley and D.Baltimore, unpublished results). Immunoprecipitation of BAN 2B and BETA cell lines with sera directed against determinants specific to the type IV protein (kindly provided by Owen Witte) showed that the overexpression is largely due to type IV protein. Clones of ΔSH and ΔSP were also active in *in vitro* kinase assay (data not shown). Two observations confirm that the proteins expressed were those predicted by the sequence of the mutant virus. First the observed mol. wts of all mutants were as expected. Secondly, while clones expressing pp160$^{v\text{-}abl}$ could be immunoprecipitated by antisera directed against both *gag* and *abl* determinants, clones ΔXB, ΔPH, and ΔSH could not be precipitated with anti-gag sera (data not shown), confirming that the transforming ability of these proteins was not due to recombination with helper virus.

Myristoylation of c-abl type IV

BETA cells overexpressing type IV and a high expresser of transforming mutant ΔSH were labeled with [^{3}H]myristic acid and immunoprecipitated with sera directed against the C-terminus. As expected from the primary sequence, c-*abl* type IV (Figure 4A, lane 1) and the ΔSH mutant (lane 3) appear to label with [^{3}H]myristic acid as efficiently as pp160$^{v\text{-}abl}$ (lane 4) given their relative expression levels (compare lanes in Figure 4A, [^{3}H]myristate label, with the corresponding lanes in Figure 4B, [^{35}S]methionine label). Since the ΔSH mutant has only the first 14 amino acids of c-*abl* type IV, this sequence appears to be sufficient for myristoylation. Thus, we expect the ΔSP, ΔPH and ΔXB mutants to be myristoylated.

Growth properties of c-abl overexpressing cell lines

Clonal cell lines representing the c-*abl* mutants were plated in soft agar as a further assay of transformation. BETA cells, which expressed the highest level of type IV protein, did not grow in soft agar. Mutant lines representing ΔSH, ΔPH, and ΔXB all grew efficiently in soft agar (Table II). In liquid culture, c-*abl* mutants ΔSH, ΔPH, and ΔXB all exhibit increased saturation density, marked morphological transformation with similar round, refractile appearance to v-*abl* transformed cells, and clear anchorage independence. Nonetheless, they grow somewhat more slowly than the NIH 3T3 cells from which they were derived. Growth curves for ΔSH, ΔPH and ΔXB mutants all show much higher saturation densities, but slower rates than parental 3T3 cells: initial doubling times were 1.5- to 2.2-fold higher for the Δ mutants. This lower growth rate is qualitatively similar to that seen in fibroblasts freshly transformed by v-*abl* (Ziegler *et al.*, 1981). BETA cells also grow significantly more slowly than parental NIH 3T3 cells (2.4-fold), whereas control cells transfected with a *neo* marker alone are unaltered in their growth properties.

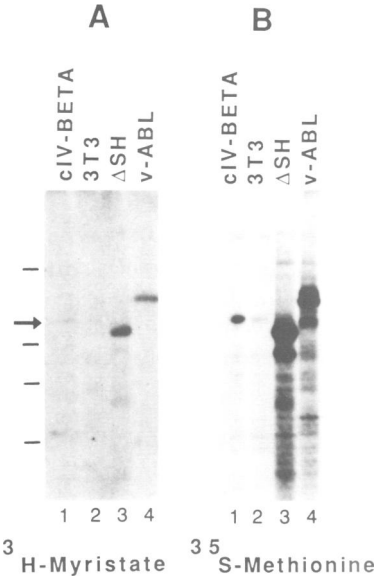

Fig. 4. Myristoylation of c-*abl* type IV. NIH 3T3 cells expressing *abl* proteins were labeled in [^{3}H]myristate and [^{35}S]methionine as described in Materials and methods. Labeled lysates were clarified and immunoprecipitated with antiserea specific to the *abl* C-terminus (PEX4/PEX5) and resolved on 7% SDS−polyacrylamide gels. (**A**) myristate labeling; (**B**) methionine labeling. **Lane 1**, clone BETA (type IV overexpresser); **lane 2**, parental NIH 3T3 cells; **lane 3**, ΔSH transformed clone (p135); **lane 4**, N54 cells (pp160$^{v\text{-}abl}$ expresser). Exposure times: **A**, 50 days; **B**, 20 h.

Table II. Transforming properties of mutant viruses and cells

	Type IV	ΔSH	ΔXB	ΔPH	ΔSP	v-*abl*
Viral titre[a] (f.f.u./ml)	N/A	5×10^5	5×10^5	5×10^5	N/A	10^6
Growth in soft agar[b]	−	++	++	++	−	+++
Bone marrow transformation[c]	N/A	−	+	+	N/A	+

[a]Focus-forming titers from clonal cell lines producing viruses expressing the mutant c-*abl* genes and M-MuLV helper.
[b]Transforming NIH/3T3 cells expressing the overexpressed wild-type or mutant protein were plated in soft agar (as described in Materials and methods), fed on days 5 and 10, and scored on day 23.
[c]Bone marrow transformation was performed on marrow from leg bones of young BALB/c females as described (Rosenberg and Baltimore, 1976). Bone marrow was grown in liquid culture, and scored positive if bulk populations could be grown long term and were B220 positive by immunofluorescence (see Materials and methods). N/A, not applicable.

Lymphoid transformation and in vivo properties of c-abl viruses

The ability of A-MuLV to transform lymphoid cells requires both the *gag* moiety (Prywes *et al.*, 1985), and the *abl*, as opposed to *src*, kinase domain (Mathey-Prevot and Baltimore, 1985). We wished to determine whether the N-terminal mutants would also transform lymphoid cells or if additional alterations were necessary. Viral supernatants from each of the transformed lines generated by co-transfection with M-MuLV helper were titered for focus formation on NIH 3T3 cells. Titers for clones representing each mutant were obtained and in each case, at least one line produced as high a titer virus ($0.5-1 \times 10^6$) as would be found with A-MuLV (Table II).

To characterize the transforming specificity of these viruses further, we infected bone marrow from BALB/c mice with mutant viruses ΔXB, ΔPH, and ΔSH (Table II). Marked transformation of bone marrow by A-MuLV, ΔPH and ΔXB viruses was seen, whereas ΔSH virus or M-MuLV gave no substantial proliferation of lymphoid cells in liquid culture. As the liquid culture assay is quite sensitive—a few transformed clones can overtake the culture—non-transformation by ΔSH is not likely to be merely an efficiency problem.

Populations of transformed lymphoid cells were subcultured from the feeder layers established in the primary cultures. These bulk cultures were of characteristic lymphoid morphology, each was B220$^+$ by immunofluorescence (a marker characteristic of Abelson transformed pre-B cells), and could be maintained in culture for months. Thus, these viruses gave standard Abelson virus lymphoid transformation *in vitro*. Portions of these bulk lymphoid populations expressing pp140$^{\Delta PH}$, and pp143$^{\Delta XB}$ were labeled with [^{32}P]orthophosphophoric acid and immunoprecipitated with anti-*abl* sera (Figure 5, lanes 2, 5) and with anti-p15 *gag* sera (lanes 3, 6) in parallel with immunoprecipitation of the same mutants in NIH 3T3 cells (lanes 1, 4) labeled in a similar manner and immunoprecipitated with anti-*abl* sera. The results show that the immortalized lymphoid cells express *abl* proteins of the same size as that expressed in the fibroblast producer lines, and that no *gag* determinants were present on the *abl* moieties in the lymphoid lines. Thus, it is unlikely that the transformation was due to a recombination event, but rather was the direct result of the transforming mutants. As such, *gag* is clearly not required for lymphoid transformation, and the earlier evidence for a *gag* requirement (Prywes *et al.*, 1985) more likely reflects the ability of several types of N-terminal modification of *abl* to support lymphoid transformation.

A more stringent test of the transforming ability of these viruses is whether they cause *in vivo* disease. Newborn BALB/c litters were injected intraperitoneally with 100 μl of viral filtrates adjusted to titers of 5×10^5 f.f.u/ml (Table III). Only N54 supernatants caused disease rapidly and efficiently (20−30 days post-infection). ΔPH virus was inefficient at inducing Abelson disease: only one of six littermates was afflicted and the latency (90 days) was quite long (nonetheless, no thymic involvement was seen). ΔSH virus gave no Abelson disease. ΔXB virus did, however, induce disease at days 45 and 60. When cells from a tumor in the day 45 mouse were cultured in media optimal for growth of B cells (Rosenberg and Baltimore, 1976), a bulk population of cells of lymphoid morphology was obtained. These cells were metabolically labeled with [^{32}P]orthophosphophoric acid and immunoprecipitated in parallel with the lymphoid cells transformed *in vitro*. The population strongly expressed the ΔXB protein (Figure 5, lane 7; compare size and lack *gag* determinants in lane 8) suggesting the ΔXB may mimic most of the properties of pp160$^{v\text{-}abl}$ in the mouse, though perhaps with lower efficiency. Further characterization of the *in vivo* properties of the ΔXB virus and the cells it transforms are in progress.

Discussion

A retroviral construct that directs synthesis of c-*abl* type IV protein does not detectably transform fibroblasts. Clones with 10-fold overexpression of the protein were isolated: they

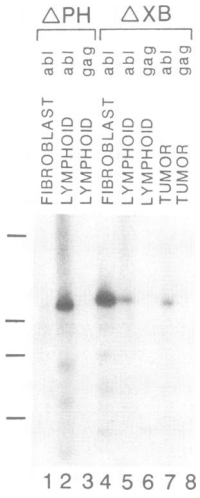

Fig. 5. Immunoprecipitation of *abl* mutant proteins from transformed lymphoid cells. *In vitro* derived bone marrow (BALB/c mice) transformants were subcultured from liquid cultures as described in the text. Subcultures were labeled in phosphate-free DME (described in Materials and methods) with [^{32}P]orthophosphate, lysed, clarified, and immunoprecipitated with sera directed against C-terminal determinants of the abl protein (PEX4/PEX5) or anti-p15 gag sera. **Lane 1**, ΔPH/NIH 3T3, anti-abl; **lane 2**, ΔPH/lymphoid, anti-abl; **lane 3**, ΔPH/lymphoid, anti-gag; **lane 4**, ΔXB/NIH 3T3, anti-abl; **lane 5**, ΔXB/lymphoid, anti-abl; **lane 6**, ΔXB/lymphoid, anti-gag; **lane 7**, ΔXB/tumor, anti-abl; **lane 8**, ΔXB-tumor, anti-gag. Equal numbers of cultured lymphoid cells were immunoprecipitated; immunoprecipitates from fibroblasts reflect about 2.5- to 5-fold fewer cells.

Table III. Effect of transforming viruses on neonates

Virus	Diseased mice/ infected mice	Day of death or autopsy of diseased animal
Ab-MuLV [N54]	4/4	d21, d26 and d27
Mo-MuLV	0/2	none
ΔXB	2/4	d45 and d60
ΔPH	1/6	d90
ΔSH	0/7	none

Newborn litters were inoculated by intraperitoneal injection with 100 μl of virus at 5×10^5 f.f.u./ml. Animals were monitored for signs of disease as described in Materials and methods. Other littermates were normal at day 120.

retain normal morphology and do not grow in soft agar. Higher expressing clones have not been isolated suggesting that further synthesis of *abl* protein may be detrimental to cell growth. In fact, the 10-fold overexpressing line BETA grows poorly. Cell lines transformed by *abl* derivatives can make even less *abl* protein than in BETA cells (e.g. ΔPH in Figure 3, lane 5), so c-*abl* IV clearly has little or no transforming ability. Similarly, c-*abl* I expressed either from a retroviral construct or an SV40-based expression system has no transforming ability (Jackson and Baltimore, unpublished results). These results with c-*abl* agree with similar experience with c-*src* and c-*fps* (Parker *et al.*, 1984; Foster *et al.*, 1985; Shalloway *et al.*, 1984), but contrast to the transforming potential of overexpressed c-*mos*, c-*ras*, and c-*fos* (Oskarsson *et al.*, 1980; Chang *et al.*, 1982; Miller *et al.*, 1984).

Certain modifications of c-*abl* IV activate its transforming potential. The efficiency of transformation by these mutants is so high that they appear qualitatively different from the non-transforming wild type c-*abl* type IV. Previous work indicated that v-*abl* requires N-terminal myristoylation to be transforming (Rein *et al.*, 1986; G.Q.Daley and D.Baltimore, unpublished results): c-*abl* IV was shown here to be myristoylated, thus its lack of transforming ability is not due to a lack of myristoylation.

The transforming ability of v-*abl* is abrogated by mutations that disrupt the protein tyrosine kinase activity (Prywes *et al.*, 1983). Thus, this activity is a crucial component of *abl*'s transforming ability. But all of the activating deletions studied are outside the kinase domain and the ΔXB and wild-type c-*abl* IV proteins have comparable kinase activity (Jackson and Baltimore, unpublished results). Thus, in thinking about why c-*abl* IV can be activated by N-terminal deletions, we have focused on ideas that do not directly involve the intrinsic kinase activity.

We found that the nested set of ΔSH, ΔPH, and ΔXB mutations activated fibroblast transforming ability. Not all modifications of the N-terminal region were activating: the ΔSP mutation was not. Oddly, the smallest deletion, ΔXB, gave the most potent virus: it transformed bone marrow cells and caused leukemia with the highest efficiency. The next largest one, ΔPH, was less efficient in leukemogenesis, and the largest, ΔSH, gave neither transformation of bone marrow cells nor leukemia. In its properties, ΔSH is like a v-*abl* derivative we made previously, a *gag*−*abl* virus that had only 14 N-terminal residues of *gag*. This virus also transformed fibroblasts, but not bone marrow cells, a deficiency apparently due to low stability of the protein in lymphoid cells (Prywes *et al.*, 1985). Thus, either ΔSH, and to a lesser extent, ΔPH, may have a similar stability problem or they lack some specific region needed for transformation of lymphoid cells. At present, the two possibilities cannot be distinguished.

The ΔXB activating deletion is in a region of the c-*abl* gene well away from the kinase domain, but one that is quite similar in structure to the N-terminus of c-*src* and other non-receptor tyrosine kinases. In fact, the G63D, R95W, and T96I c-*src* activating mutations (Kato *et al.*, 1986), as well as an activating 90−95 c-*src* deletion (Potts *et al.*, 1988) are near or within a region homologous to the ΔXB deletion. Furthermore, N-terminal changes in v-*src* can modulate its transforming ability, including its cell type-specificity (Jove and Hanafusa, 1987; Poirier *et al.*, 1987). A similar amino acid stretch is also found in phospholipase C-type II (Stahl *et al.*, 1988) and in the v-*crk* oncogene (Mayer *et al.*, 1988).

Figure 6 summarizes the topology of the major homology domains among the cytoplasmic tyrosine kinases and phospholipases. The SH1 (*src* homology 1) comprises the kinase domain and clearly participates in the transforming ability of *src* and *abl*. SH2 was a designation suggested by Pawson and co-workers (Sadowski *et al.*, 1986) for a regulatory region immediately N-terminal to the kinase domain and roughly coincident with one of the homologies suggested by Mayer *et al.* (1988). From the deletional studies of Prywes *et al.* (1983, 1985) it is evident that SH2 is required for the *abl* kinase to be a transforming protein. SH3 is defined both by the second homology suggested by Mayer and by the ΔXB mutation. Its modification activates transforming potential in the normal cellular *src* and *abl* genes.

We interpret the ΔXB mutation as one that removes a

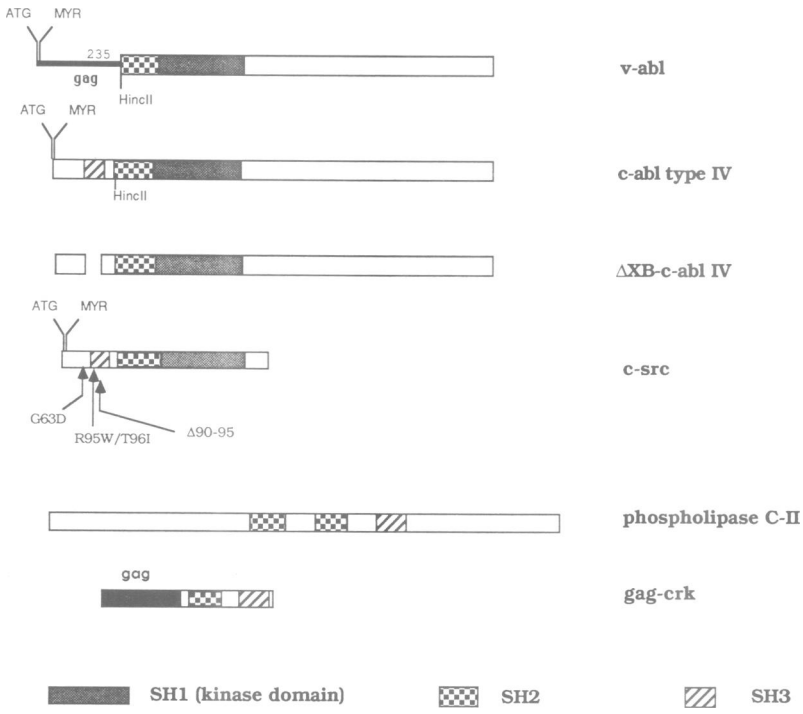

Fig. 6. Summary of SH2 and SH3 homology domains of cytoplasmic tyrosine kinases and phospholipases. Shown are the structures of $p160^{gag\text{-}v\text{-}abl}$ (Lee *et al.*, 1984), $p150^{c\text{-}abl\ IV}$ (Ben-Neriah *et al.*, 1985), $p143^{c\text{-}abl\ IV\text{-}\Delta XB}$, $p60^{c\text{-}src}$ (Takeya and Hanafusa, 1982), $p47^{gag\text{-}crk}$ (Mayer *et al.*, 1988), and phospholipase C-II (Stahl *et al.*, 1988) (Alignments of the detailed sequences are shown in Mayer *et al.*, 1988 and Stahl *et al.*, 1988). Note the deletion of the SH3 homology in $p160^{gag\text{-}abl}$ and the ΔXB mutant.

segment of protein that interacts with another protein (or molecule) and that the interacting protein ordinarily blocks the inherent transforming ability of c-*abl* IV. The SH3 sequence relationship among *abl*, *src*, *crk* and phospholipase C-II may arise from a common interaction of a class of regulatory proteins with each of these proteins. In one sense, the gene for such a modulatory protein would be an anti-oncogene: homozygous inactivation of such a gene would be expected to activate cellular transformation. Presumably the ordinary function of this interacting protein relates to the normal function of c-*abl*. It could be a protein that controls substrate interaction of the kinase either by mediating substrate recognition or by directing localization of *abl* relative to its substrates. It could alternatively mediate interaction with a phosphatase, or with a protein that sends a signal to *abl*, as a G protein might. Direct biochemical comparison of cells overexpressing type IV and its activated mutants and a more refined mutational analysis may make the identification of such interacting proteins possible.

Materials and methods

Plasmid constructions

c-*abl* sequences derive from reconstructed cDNAs from a type IV cDNA derived from a 70Z/3 cDNA library (Ben-Neriah *et al.*, 1986) for 5′ sequences and an oligo-dT primed 70Z library for the 3′ sequences (A.Bernards and D.Baltimore, unpublished results). The vector used is a simple retroviral vector analogous to v-*abl*: MoMuLV LTRs and mouse flanking sequences are cloned into a pUC13 backbone (Mathey-Prevot and Baltimore, 1985). Constructions are as follows:

(i) pAB160: as described previously (Goff *et al.*, 1981).

(ii) pPLBAN: A type IV c-*abl* cDNA was truncated at a BanI site immediately 5′ to the presumed ATG. This site was end-filled with Klenow and fitted with *Eco*RI linkers. A 440 bp fragment from this synthetic *Eco*RI site to the *Hin*cII site homologous to that near the *gag*/v-*abl* junction was cloned into the *Eco*RI and *Hin*cII sites of a pUC13 vector lacking a *Pst*I site. This vector, p5′IV, was used for subsequent mutagenesis. The *Eco*RI−*Hin*cII 440 bp fragment was used to replace a similar fragment encoding a type I cDNA in vector pPLCI (Jackson and Baltimore, unpublished results). A three part ligation was done: a 440 bp *Eco*RI−*Hin*cII type IV specific fragment, an 1863 bp *Hin*cII−*Xho*I fragment spanning the c-*abl* kinase domain, and a 6 kb *Xho*I−*Eco*RI fragment comprising the c-*abl* C terminus, viral LTR's and pUC13 backbone.

(iii) pPLΔSH: A similar three-part ligation was performed, using a 68 bp *Eco*RI−*Stu*I fragment in place of the 440-bp fragment. The *Stu*I−*Hin*cII blunt ligation maintains the reading frame; this was verified by Maxam−Gilbert (1980) sequencing.

(iv) pPLΔPH: p5′IV was cut with *Pst*I, treated with mung-bean nuclease to remove the 3′ overhang, and fitted with *Spe*I 10mer linkers, cut with *Spe*I and recircularized, recreating the *Hin*cII site. The 170 bp *Eco*RI−*Hin*cIII fragment was used in a similar three part ligation. Reading frame was verified by sequencing.

(v) pPLΔXB: p5′IV was cut with *Xmn*I and *Bal*I. The 1.2 kb *Xmn*I fragment was ligated to the 2.2 kb *Xmn*I−*Bal*I fragment, and screened for ampicillin resistance to ensure reconstruction of the amp^r gene. The *Eco*RI−*Hin*cII fragment from this mutant was used as above.

(vi) pPLΔSP: p5′IV was cut with *Pst*I, treated with mung-bean nuclease to remove the 3′ overhang, fitted with *Xho*I 8mer linkers, cut with *Xho*I and recircularized. The 350 bp *Eco*RI−*Hin*cII fragment was used in a similar three part ligation. All DNA modifying enzymes and linkers were obtained from New England Biolabs.

Cells, viruses, mice and DNAs

NIH/3T3 fibroblasts and derived lines were grown in Dulbecco's modified Eagles medium (DME) with 10% calf serum. A-MuLV titers were determined by focus formation on NIH/3T3 cells (Scher and Siegler, 1975). *In vitro* lymphoid cell transformation in agar and liquid has been described (Rosenberg *et al.*, 1975; Rosenberg and Baltimore, 1978). Transfections and DNA preparation as described (Prywes *et al.*, 1983).

Newborn litters of BALB/c mice were inoculated by intraperitoneal injection with 100 μl of virus at 5×10^5 f.f.u./ml as described (Rosenberg and Baltimore, 1976). Animals were monitored for signs of disease. If characteristic disease ('caput', shivering, cachexia, hunched shoulders) was

observed, autopsy was performed, or in some cases, the animal died before autopsy. Autopsy focuses on characteristic sets of lymph node tumors, spleen size and color, thymus size and color, and any obvious tumors. Abelson disease was scored positive if superficial symptoms, lymph node tumours and splenomegaly were positive and the thymus was normal.

Labeling, immunoprecipitation and kinase assays

For labeling transformed fibroblasts with $^{32}PO_4^{-2}$, cells were plated at 4 $\times$ 10^6 cels/6 cm dish. Cells were rinsed in phosphate-free DME and labeled in 1 ml of phosphate-free DME/2% dialysed calf-serum/1 mCi $^{32}PO_4^{-2}$ (New England Nuclear) for 2–4 h. Lymphoid cells were labeled similarly in liquid culture at 2–5 $\times$ 10^6/ml.

Cells were labeled with [^{35}S]methionine (New England Nuclear) in methionine-free DME/5% calf serum for 2 h at 200 μCi/ml after 1 h growth in methionine-minus DME.

Cells were lysed in 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM Tris, pH 7.5, 1 mM EDTA, 1% Aprotinin (Sigma), 1 mM PMSF, clarified at 13 000 *g* for 15 s. Often a second clarification at 100 000 *g* for 10 min was performed. Samples were normalized for trichloroacetic acid precipitable counts prior to immunoprecipitation, which proved to be essential because slower growing overexpressors, such as BETA cells, take up much less label. Immunoprecipitations used 1:1 cocktails of PEX4/PEX5 (Konopka *et al.*, 1984; Konopka and Witte, 1985). Anti-*gag* sera from NCI was derived from sera directed against AKR virus p15 protein. Samples were resolved on 7% SDS–polyacrylamide gels.

Kinase assays were as described (Konopka *et al.*, 1984; Konopka and Witte, 1985).

Acknowledgements

We thank Andre Bernards, George Daley and Bernard Mathey-Prevot for helpful advice and DNAs, and Owen Witte for antisera. This work was supported by grant #CA38497 from the National Cancer Institute.

References

Abelson,H.T. and Rabstein,L.S. (1970) *Cancer Res.*, **30**, 2213–2222.
Baltimore,D., Rosenberg,N. and Witte,O.N. (1979) *Immunol. Rev.*, **48**, 3–22.
Ben-Neriah,Y., Bernards,A., Paskind,M., Daley,G.Q. and Baltimore,D. (1986) *Cell*, **44**, 577–586.
Bernards,A., Rubin,C.M., Westbrook,C.A., Paskind,M. and Baltimore,D. (1987) *Mol. Cell. Biol.*, **7**, 3231–3236.
Bernards,A., Paskind,M. and Baltimore,D. (1988) *Oncogene*, **2**, 297–304.
Bishop,J.M. (1983) *Annu. Rev. Biochem.*, **52**, 301–354.
Chang,E.H., Furth,M.E., Scolnick,E.M. and Lowy,D.R. (1982) *Nature*, **297**, 479–483.
Daley,G.Q., McLaughlin,J., Witte,O.N. and Baltimore,D. (1987) *Science*, **237**, 532–535.
Downward,J., Yarden,Y., Mayes,E., Scrace,G., Totty,N., Stockwell,P., Ullrich,A., Schlessinger,J. and Waterfield,M.D. (1984) *Nature*, **311**, 483–485.
Foster,D.A., Shibuya,M. and Hanafusa,H. (1985) *Cell*, **42**, 105–115.
Fry,M.J., Gebhardt,A., Parker,P.J. and Foulkes,J.G. (1985) *EMBO J.*, **4**, 3173–3178.
Goddard,J.M., Weiland,J.J. and Capecchi,M.R. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 2172–2176.
Goff,S.P., Gilboa,E., Witte,O.N. and Baltimore,D. (1980) *Cell*, **22**, 777–785.
Goff,S.P., Witte,O.N., Gilboa,E., Rosenberg,N. and Baltimore,D. (1981) *J. Virol.*, **38**, 460–468.
Goff,S.P., Tabin,C.J., Wang,Y.-J., Weinberg,R. and Baltimore,D. (1982) *J. Virol.*, **41**, 271–285.
Heisterkamp,N., Stephenson,J.R., Grofften,J., Hansen,P.F., de Klein,A., Bartram,C.R. and Grosveld,G. (1983) *Nature*, **306**, 239–242.
Hoffman-Falk,H., Einat,P., Shilo,B. and Hoffman,F.M. (1983) *Cell*, **32**, 589–598.
Hunter,T. and Sefton,B.M. (1980) *Proc. Natl. Acad. Sci. USA.*, **77**, 1311–1315.
Iba,H., Takeya,T., Cross,F.R., Hanafusa,T. and Hanafusa,H. (1984) *Proc. Natl. Acad. Sci. USA.*, **81**, 4424–4428.
Jove,R., Mayer,B.J., Iba,H., Laugier,D., Poirier,F., Calothy,G., Hanafusa,T. and Hanafusa,H. (1986a) *J. Virol.*, **60**, 840–848.
Jove,R., Garber,E.A., Iba,H. and Hanafusa,H. (1986b). *J. Virol.*, **60**, 849–857.
Jove,R. and Hanafusa,H. (1987) *Annu. Rev. Cell. Biol.*, **3**, 31–56.
Kaplan,J.M., Mardon,G., Bishop,J.M. and Varmus,H.E. (1988) *Mol. Cell. Biol.*, **8**, 2435–2441.
Kato,J.-Y., Takeya,T., Grandori,C., Iba,H., Levy,J.B. and Hanafusa,H. (1986) *Mol. Cell. Biol.*, **6**, 4155–4160.
Konopka,J.B., Davis,R.L., Watanabe,S.M., Ponticelli,A.S., Schiff-Maker,L., Rosenberg,N. and Witte,O.N. (1984) *J. Virol.*, **51**, 223–232.
Konopka,J.B. and Witte,O.N. (1985) *Mol. Cell. Biol.*, **5**, 3116–3123.
Kozak,M. (1986) *Cell*, **48**, 283–292.
Lee,R., Paskind,M., Wang,J.Y.J. and Baltimore,D. (1984) In Weiss,R., Teich,N., Varmus,H. and Coffin,J. (eds), *RNA Tumor Viruses*. Cold Spring Harbor Laboratory, Vol. II, pp. 861–868.
Levy,J.B., Iba,H. and Hanafusa,H. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 4228–4232.
Mathey-Prevot,B. and Baltimore,D. (1985) *EMBO J.*, **4**, 1769–1774.
Mathey-Prevot,B. and Baltimore,D. (1988) *Mol. Cell. Biol.*, **8**, 234–240.
Maxam,A. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
Mayer,B.J., Hamaguchi,M. and Hanafusa,H. (1988) *Nature*, **332**, 272–275.
Miller,A.D., Curran,T. and Verma,I.M. (1984) *Cell*, **36**, 51–60.
Oskarsson,M., McClements,W.L., Blair,D.G., Maizel,J.V. and Vande Woude,G.F. (1980) *Science*, **207**, 1222–1224.
Parker,R.C., Varmus,H.E. and Bishop,J.M. (1984) *Cell*, **37**, 131–139.
Poirier,F., Laugier,D., Marx,M., Dambrine,G., Garber,E.A., Genvrin,P., David-Pfeuty,T. and Calothy,G. (1987) *J. Virol.*, **61**, 2530–2539.
Ponticelli,A.S., Whitlock,C.A., Rosenberg,N. and Witte,O.N. (1982) *Cell*, **29**, 953–960.
Potts,W.M., Reynolds,A.B., Lansing,T.J. and Parsons,J.T. (1988) *Oncogene Res.*, in press.
Prywes,R., Foulkes,J.G., Rosenberg,N. and Baltimore,D. (1983) *Cell*, **34**, 569–579.
Prywes,R., Hoag,J., Rosenberg,N. and Baltimore,D. (1985) *J. Virol.*, **54**, 123–132.
Rein,A., McClure,M.R., Rice,N.R., Luftig,R.B. and Schultz,A.M. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 7246–7250.
Reynolds,F.H., van de Ven,W.J.M. and Stephenson,J.R. (1980) *J. Virol.*, **36**, 374–386.
Rosenberg,N., Baltimore,D. and Scher,C.D. (1975) *Proc. Natl. Acad. Sci. USA.*, **72**, 1932–1936.
Rosenberg,N. and Baltimore,D. (1976) *J. Exp. Med.*, **143**, 1453–1463.
Rosenberg,N. and Baltimore,D. (1978) *J. Exp. Med.*, **147**, 1126–1141.
Rosenberg,N.E. and Witte,O.N. (1980) *J. Virol.*, **33**, 340–348.
Sadowski,I., Stone,J.C. and Pawson,T. (1986) *Mol. Cell. Biol.*, **6**, 4396–4408.
Scher,C.D. and Siegler,R. (1975) *Nature*, **253**, 729–731.
Sefton,B.M., Trowbridge,I.S., Cooper,J.A. and Scolnick,E.M. (1982) *Cell*, **31**, 465–474.
Shalloway,D., Coussens,P.M. and Yaciuk,P. (1984) *Proc. Natl. Acad. Sci. USA.*, **81**, 7071–7075.
Sherr,C.J., Rettenmier,C.W., Sacca,R., Roussel,M.F., Look,A.T. and Stanley,E.R. (1985) *Cell*, **41**, 665–676.
Shtivelman,E., Lifshitz,B., Gale,R.P., Roe,B.A. and Canaani,E. (1986) *Cell*, **47**, 277–284.
Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 327–341.
Stahl,M.L., Ferenz,C.R., Kelleher,K.L., Kriz,R.W. and Knopf,J.L. (1988) *Nature*, **332**, 269–272.
Takemori,T., Miyazoe,I., Shirasawa,T., Taniguchi,M. and Graf,T. (1987) *EMBO J.*, **6**, 951–956.
Takeya,T. and Hanafusa,H. (1982) *J. Virol.*, **44**, 12–18.
Towler,D.A., Eubanks,S.R., Towery,D.S., Adams,S.P. and Glaser,L. (1987) *J. Biol. Chem.*, **262**, 1030–1036.
Ushiro,H. and Cohen,S. (1980) *J. Biol. Chem.*, **255**, 8363–65.
Wang,J.Y.J. and Baltimore,D. (1983) *Mol. Cell. Biol.*, **3**, 773–779.
Witte,O.N., Rosenberg,N.E. and Baltimore,D. (1979) *Nature*, **238**, 826–831.
Witte,O.N., Dasgupta,A. and Baltimore,D. (1980) *Nature*, **283**, 826–831.
Yarden,Y., Escobedo,J.A., Kuang,W.-J., Yang-Feng,T.L., Daniel,T.O., Tremble,P.M., Chen,E.Y., Harkins,R.N. Francke,U., Fried,V.A., Ullrich,A. and Williams,L.T. (1986) *Nature*, **323**, 226–232.
Ziegler,A.F., Whitlock,C.A., Goff,S.P., Gifford,A. and Witte,O.N. (1981) *Cell*, **27**, 477–486.

Received on October 31, 1988; revised on December 6, 1988