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The Minimum Transforming Region of v-*abl* Is the Segment Encoding Protein-Tyrosine Kinase

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Only 1.2 kilobases (kb) at the 5′ end of the 3.9-kb v-*abl* sequence in Abelson murine leukemia virus is required for fibroblast transformation. A precise delineation of this minimum transforming region was made by using small 5′ or 3′ deletions. Insertions of four amino acids, generated by putting synthetic DNA linkers into various restriction enzyme cleavage sites, abolished transforming activity, indicating that much of the internal sequence of the minimum transforming region plays a critical role in the transformation process. This 5′ 1.2 kb of v-*abl* encodes protein-tyrosine kinase activity when expressed in *Escherichia coli*. Each of the mutations which caused a loss of transformation activity also resulted in a loss of protein-tyrosine kinase activity when expressed in *E. coli*. The minimum transforming region of v-*abl* contains amino acid homology to other protein-tyrosine kinase oncogenes, and a comparison with these oncogenes is presented.

Abelson murine leukemia virus (A-MuLV) is a replication-defective retrovirus capable of transforming fibroblast cells in vitro (28) and lymphoid cells both in vitro and in vivo (1, 25, 26). The viral genome encodes a single protein, the gene for which was produced as a result of a fusion between the Moloney MuLV (M-MuLV) *gag* gene and cell-derived v-*abl* sequences (11, 24, 32, 38, 44). The largest variant of A-MuLV encodes a 160-kilodalton (kd) protein, of which 130 kd is determined by the v-*abl* sequence, but only the N-terminal 45 kd of the v-*abl*-encoded protein sequence is required for fibroblast transformation (22). Though nearly all of the *gag* sequence can be deleted without abolishing fibroblast transformation, much of *gag* is essential for lymphoid transformation (22).

The v-*abl* gene, along with *src* and several other oncogenes, encode proteins that display tyrosine-specific protein kinase activity (13, 41). In addition, these various oncogene products have a region of strongly conserved amino acid sequence (3, 23, 31). In the v-*abl*-encoded protein, this homology region resides within the N-terminal 45 kd, a segment we will refer to as the minimum transforming region. J. J.-Y. Wang and D. Baltimore (J. Biol. Chem., in press) expressed this region in *E. coli* and found that its product displays protein-tyrosine kinase activity, demonstrating that the protein-tyrosine kinase domain and minimum transforming region are contained within the same 1.2-kilobase (kb) fragment of v-*abl* sequence. Witte et al. (42) showed that a transformation-defective mutant, A-MuLV[P92*td*], contains a deletion within this region and lacks tyrosine-specific in vitro autophosphorylation activity. Srinivasan et al. (34) also tested plasmids cut within this region and found them to be transformation defective.

We have now further tested the requirement for the minimum transforming region by constructing specific mutants. Small 5′ or 3′ deletions were made to examine the outer boundaries. Insertions of four amino acids at various points were made to test for the necessity of internal sequences. We found that nearly all the mutations within the minimum transforming region result in transformation-defective genomes. The mutant genes were expressed in *Escherichia coli* and assayed for protein-tyrosine kinase activity. The loss of transforming ability correlated with a loss of tyrosine-specific protein kinase activity.

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MATERIALS AND METHODS

Cells and viruses. Normal and A-MuLV-transformed NIH/3T3 fibroblasts were grown in Dulbecco modified Eagle's medium with 10% calf serum. Transfections by the calcium phosphate method were performed essentially as described (40) and modified (22).

Bacterial labeling. [^{35}S]methionine metabolic labeling and $^{32}P_i$ in vivo pulse-labeling of *E. coli* HB101 containing the various expression plasmids were performed essentially as described (39). For [^{35}S]methionine labeling, bacteria were grown in minimal medium containing all the amino acids except methionine. An overnight culture was diluted 1:40 into fresh methionine-free medium and incubated for 2 h at 30°C. The culture was then shifted to 40°C for 1 h. [^{35}S]methionine was then added to 100 μCi/ml, and the bacteria were incubated for 1 h at 40°C. The bacteria were then collected by centrifugation and resuspended in 0.5 ml of phospholysis buffer (10 mM sodium phosphate [pH 7.5], 100 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and 2 mM EDTA). The sample was sonicated twice for 15 s and centrifuged at 13,000 × *g* for 15 min. Three microliters of anti-AbT serum (a mouse serum from an animal that had rejected an A-MuLV-induced tumor [43]) was added to the supernatant and incubated on ice for 1 h. Twenty-five microliters of a 50% protein A–Sepharose suspension was then added and incubated on ice for 30 min. The immune complexes were collected by centrifugation and washed four times in phospholysis buffer and two times in water. The complexes were then resuspended in 50 μl of SDS buffer (67 mM Tris-hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 5 mM EDTA, 20 mM dithiothreitol, and bromophenol blue). This solution was boiled for 5 min and fractionated by electrophoresis through an SDS–10% polyacrylamide gel as described elsewhere (44). Samples were normalized for trichloroacetic acid-precipitable radioactivity before immunoprecipitation. The final gel was treated with En^{3}Hance (New England Nuclear Corp.) and developed by fluorography.

For in vivo labeling of *E. coli* with $^{32}PO_4^{2-}$, bacteria were grown overnight at 30°C in L broth. This culture was diluted 1:40 into fresh medium, grown to mid-log phase (A_{650} = 0.3) at 30°C, and shifted to 40°C for 2 h. Samples were then normalized for cell number, collected by centrifugation, and washed twice in 50 mM Tris-hydrochloride (pH 7.5)–100 mM NaCl–10 mM $MgCl_2$. The bacteria were then resuspended in the same buffer at 3×10^9 cells per ml ($A_{650} = 0.1 = 8 \times 10^7$ cells per ml). These steps were all performed at 4°C. Five microliters of 300 μCi of $^{32}PO_4^{2-}$ per ml was added to 45 μl of the above bacterial suspension and incubated at 40°C for 15 min. Twenty five microliters of 3× SDS buffer was added, and the bacteria were lysed by boiling for 10 min. Twenty-five microliters was then electrophoresed through an SDS–10% polyacrylamide gel. The gel was fixed in 20% isopropanol–10% acetic acid for longer than 30 min and then soaked in 1 N NaOH at 50°C for 1 h. This gel was washed twice quickly with water and soaked in 10% acetic acid for longer than 30 min. Finally the gel was dried down and developed by autoradiography with an intensifying screen.

Plasmid construction. (i) pR1. The circularly permuted P160 coding plasmid, pAB160 (17), was modified to remove the *Eco*RI site in the pBR322 backbone. This was done by the following scheme: (i) digesting with *Eco*RI, (ii) filling in the cohesive ends with the Klenow fragment of *E. coli* polymerase I, (iii) blunt-end ligating to recircularize, (iv) digesting with excess *Eco*RI to select against plasmids still retaining an *Eco*RI site, (v) transforming *E. coli* to ampicillin resistance, and (vi) screening colonies for intact plasmids lacking the *Eco*RI site. DNA was prepared rapidly from the colonies by the method of Ish-Horowicz and Burke (15) and digested with the appropriate diagnostic restriction enzymes.

The plasmid pR1 was formed by inserting 12 base pairs (bp) at the first *Hin*cII site in the v-*abl* region of an *Eco*RI-less plasmid. The 12-bp unit was a synthetic piece of DNA containing an *Eco*RI site; we will refer to this unit as an (*Eco*RI)linker. Because there are four *Hin*cII sites in the plasmid, (*Eco*RI)linkers were placed randomly, and the appropriate plasmid was selected by screening their structures. To this end the plasmid was partially digested with *Hin*cII, producing blunt ends, and linear DNA was isolated from an agarose gel by using glass powder (37). (*Eco*RI)linkers were ligated to this DNA overnight at 15°C. The sample was then phenol extracted once, ether extracted twice, and ethanol precipitated. The DNA was digested with *Eco*RI to remove excess linkers and again phenol and ether extracted and ethanol precipitated. This DNA was ligated to recircularize it and used to transform *E. coli* to ampicillin resistance. Colonies were screened for intact plasmids containing the linker in the desired position.

(ii) pB1. pR1 was used to generate pB1. pR1, with an (*Eco*RI)linker at what was formerly the first *Hin*cII site of v-*abl*, was digested with *Eco*RI. After phenol and ether extraction and ethanol precipitation, the DNA was treated with Bal 31 exonuclease and titrated to remove ca. 100 bp from each end. The reaction was terminated with 50 mM EDTA, phenol and ether extracted, and ethanol precipitated. The DNA was then treated with the Klenow fragment of *E. coli* DNA polymerase I to blunt any single-stranded ends. (*Eco*RI)linkers were then ligated on. After further extractions and ethanol precipitations, the DNA was digested with *Eco*RI, and full-length DNA was electrophoretically purified away from excess linkers. This DNA was ligated to recircularize it and used to transform *E. coli* to ampicillin resistance. The colonies were screened for small deletions around the *Hin*cII site. pB1 was sequenced on both strands by the method of Maxam and Gilbert (19) and found to contain an in-frame deletion of 231 bp, removing the last 49 amino acids of *gag* and the first 28 amino acids of v-*abl* and leaving at the junction four amino acids encoded by the linker (Pro-Glu-Phe-Gly).

(iii) pABS. pABS was derived from a proviral P160-encoding clone, pABpv (22). pABpv was partially digested with *Sac*I, and single-cut linear DNA was electrophoretically purified. This DNA was treated with the Klenow fragment to remove the cohesive ends. The enzyme was heat killed at 68°C for 10 min. The DNA was blunt-end ligated to recircularize it and used to transform *E. coli* to ampicillin resistance. The colonies were screened for intact plasmids lacking the particular *Sac*I site of interest due to the removal of the 4-bp cohesive ends. This puts the coding region out of frame at this point.

(iv) pPs. pPS was made similarly to pAB-p (22). A retroviral vector, pMSVgpt (20), was used. Once the *gpt* gene was removed, a *Bgl*II site was left where the first *Pst*I site of M-MuLV was. The analogous *Pst*I site of A-MuLV was used. To prepare pPS, a *Pst*I-to-*Sac*I fragment was electrophoretically purified. The cohesive ends were removed with the Klenow fragment of DNA polymerase I, and 12-bp (*Bam*HI)linkers were ligated on. pMSVgpt was digested with *Bgl*II and treated with calf intestinal phosphatase. The *Bgl*II fragment (the entire plasmid without the *gpt* gene) was gel purified and ligated to the A-MuLV fragment described above (*Bam*HI and *Bgl*II have compatible cohesive ends). Ampicillin-resistant colonies were screened for the insert in the correct orientation.

(v) Linker DNA insertions. Either pAB160 (lacking an *Eco*RI site as described above) or pABX was used for insertion of 12-bp DNA segments. pABX codes for a 100-kd transforming Abelson protein (22; see Fig. 1). The plasmids were digested partially with restriction enzyme *Hae*III and fractionated by electrophoresis through an agarose gel to isolate singly cut, linear DNA. Linker DNA was ligated onto this linear DNA overnight at 15°C. (*Eco*RI)linkers 12 bp in length were used for pAB160; 12-bp (*Bam*HI)linkers were used for pABX. After ligation of the linkers, the DNA was phenol and ether extracted and ethanol precipitated. Excess linkers were subsequently removed by digestion with the appropriate restriction enzyme and fractionation by electrophoresis through an agarose gel. Linear DNA was purified from the gel, ligated to close the circles, and used to transform *E. coli* to ampicillin resistance. Colonies were screened for linker restriction sites within the minimum transforming region. The names of the plasmids generated and the position of the linker insertions are listed below (see Fig. 2).

(vi) ptX. ptX was generated from p*t(b)abl*130, kindly provided by Jean Wang. She placed a unique *Bam*HI site within the small t region of the fusion protein encoded by p*tabl*130 (Wang and Baltimore, in press). This plasmid was further modified to remove the *Eco*RI site of pBR322 in the same way as described above. To change the *Bam*HI site to an *Eco*RI site, the resultant plasmid was digested with *Bam*HI and treated with the Klenow fragment, and (*Eco*RI)linkers were ligated on. After extractions and ethanol precipitation the DNA was digested with *Eco*RI and *Sal*I. A fragment containing *Eco*RI and *Sal*I cohesive ends was electrophoretically purified, the *Eco*RI site then being where the *Bam*HI site was in p*t(b)abl*130, leaving 58 amino acids of simian virus 40 small t coding sequence. The *Sal*I site is in pBR322. An *Eco*RI-to-*Xho*I fragment was purified from pR1,

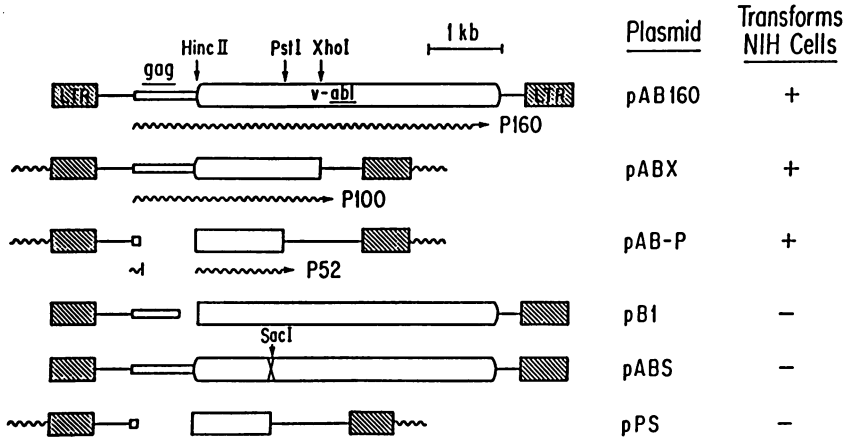

FIG. 1. Genomes used to delineate the extent of the minimum transforming region. The structure of different A-MuLV genomes are shown, along with the names of the corresponding plasmids and their ability to transform NIH/3T3 cells upon transfection with helper virus (M-MuLV) DNA. The protein products are indicated with wavy lines, along with certain relevant restriction sites. Gaps denote deletion of these sequences in the corresponding plasmid. X, The *Sac*I site in pABS was destroyed by removal of the 4-bp cohesive ends, putting the coding region out of frame at this point.

the *Eco*RI site being where the first *Hin*cII site of v-*abl* was (see above). This fragment corresponds to the 5′ 1.8 kb of v-*abl*. The two fragments described above were ligated together (*Xho*I and *Sal*I have compatible cohesive ends) and used to transform *E. coli* to ampicillin resistance. The resultant plasmid is diagrammed below (see Fig. 4).

(vii) ptB1. ptB1 was made exactly as above, except that pB1 was used instead of pR1.

(viii) ptXB1, ptXB3, ptXB4, ptXB5. ptXB1, ptXB3, ptXB4, and ptXB5 were generated as above, except that pXB1, pXB3, pXB4, or pXB5 was used instead of pR1. These plasmids were digested with *Hin*cII, and (*Eco*RI)linkers were ligated on at this site. The DNA was subsequently digested with *Eco*RI and *Xho*I and electrophoretically purified to generate a fragment similar to that described above, except for the linker insertion mutation in each of the plasmids.

(ix) ptH1. pt(*b*)*abl*130 was digested with *Bam*HI and *Xho*I to generate the backbone fragment. pH1 was digested with *Hin*cII, and 10-bp (*Bam*HI)linkers were ligated on at this point. The DNA was digested with *Bam*HI and *Xho*I to generate a fragment similar to those above, yet containing the *Eco*RI linker insertion present in pH1. These two gel purified DNAs were ligated together to make ptH1.

All the plasmids described under subheads vi through ix were constructed to retain the coding region of the small t-v-*abl* fusion protein in frame.

All restriction enzymes and Bal 31 exonuclease were obtained from New England Biolabs except for *Eco*RI. *Eco*RI, calf intestinal phosphatase, and the Klenow fragment of *E. coli* DNA polymerase I were from Boehringer Mannheim Biochemicals. Linker DNA and T4 DNA ligase was obtained from Collaborative Research, Inc.

RESULTS

Delineation of the minimum transforming region. We have previously shown that the 5′ 1.2 kb of v-*abl*, when expressed as a fusion protein with the N-terminal 34 amino acids of *gag* (plasmid pAB-P; Fig. 1), is sufficient for fibroblast transformation by A-MuLV (22). To further define the limits of this region we made small deletions at the 5′ and 3′ ends and tested the ability of these plasmids to transform NIH/3T3 cells.

For the 5′ deletions we used Bal 31 exonuclease to generate a small deletion in a P160-coding clone (see above for details of construction). This plasmid, pB1 (Fig. 1), was sequenced across the deletion site and was found to contain an in-frame deletion that removed the first 28 amino acids of v-*abl*, as well as the last 49 amino acids of *gag*. This region of *gag* is not required for fibroblast transformation (22). Plasmid pB1 did not cause transformed foci when transfected onto NIH/3T3 cells along with helper virus DNA. The helper virus DNA greatly increases the efficiency of focus formation after transfection with A-MuLV DNA (11, 12). Use of the helper means that foci are formed by spread of virus from the initially transfected cell. Thus, even this small 5′ deletion removes sequence required for transformation.

At the 3′ end, a termination mutation was made at the *Sac*I site 159 bp 5′ of the *Pst*I site used to make the transforming plasmid, pAB-P (Fig. 1). The mutated plasmid, pABS, had its *Sac*I site destroyed by blunting the cohesive ends (see above for details). This resulted in putting the coding region out of frame at this point. A similar plasmid, pPS, was constructed which had only the sequence up to the *Sac*I site (see above). Neither pABS nor pPS transformed NIH/3T3 cells upon transfection with helper virus DNA. The 3′ end of the minimum transforming region is therefore between the *Pst*I and *Sac*I sites.

Linker insertion mutations in v-*abl*. To probe which parts of the internal sequence in the minimum transforming region of v-*abl* might be dispensable, we constructed a series of mutant plasmids containing 12-bp insertions within this region. These insert four amino acids while retaining the coding region in frame. The mutations were generated by partial digestion of plasmids with the restriction enzyme *Hae*III to generate linear DNA. *Hae*III recognizes 10 sites within the minimum transforming region, so that a partial digest generating linear DNA should result in the random cleavage at one of these sites. Linear DNA was purified from agarose gels, and 12-bp units containing restriction enzyme cleavage sites (linkers) were ligated onto this DNA. The mutations were generated in two sets by utilizing either plasmid pAB160 or pABX. For pAB160, 12-bp (*Eco*RI) linkers were used; for pABX, 12-bp (*Bam*HI)linkers were used. In the final step, the DNA was ligated to close the circles and used to transform *E. coli* to ampicillin resistance

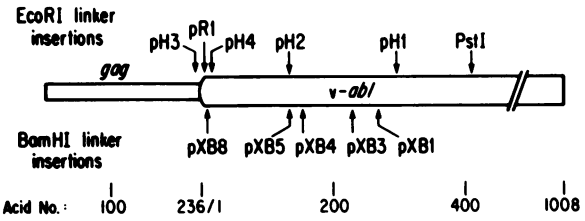

FIG. 2. Position of linker insertions. The coding region of the A-MuLV protein is diagrammed. The positions of (*Eco*RI) or (*Bam*HI)linker insertions in particular mutant plasmids are indicated. The *Pst*I restriction site marks the 3′ boundary of the minimum transforming region. The amino acid number positions of P160 are indicated by using 1 through 236 for the *gag* section of the protein, followed by 1 through 1008 for v-*abl*.

(see above for more details). Because *Hae*III digestion leaves blunt ends, the above process resulted in insertion of only the 12-bp linker unit. We sequenced across each of the linker insertion mutation sites to confirm their position and sequence. As expected, all mutants contained the 12-bp linker DNA insertion within an *Hae*III site. In all cases the coding region remained in frame.

The plasmids generated and the position of their linker insertions are indicated in Fig. 2. In a separate construction an (*Eco*RI)linker was inserted at the *Hin*cII site near the beginning of v-*abl* to produce pR1. The mutant plasmids were transfected, along with helper virus DNA, into NIH/3T3 cells. The plasmids were easily scored to be either as transforming as their parental plasmids (100 to 500 foci per μg of DNA) or nontransforming (no foci) (Table 1). The one exception was plasmid pH2 [containing an (*Eco*RI)linker insertion], which had a 5- to 10-fold-lower transfection efficiency than the parental pAB160. As a rule, insertions near the 5′ end had no effect; insertions within the minimum transforming region abolished transforming activity.

Transformed foci were picked from the plates transformed by plasmids pH4, pH3, and pH2 [all derived from pAB160 and containing an (*Eco*RI)linker]. Each of these lines expressed the expected 160-kd Abelson protein as detected by in vitro autophosphorylation reactions in immunoprecipitates (data not shown). Cellular DNA was made from these lines and probed for the mutant viral genomes. As expected, pH4- and pH3-transformed lines contained full-length viral genomes, as well as the *Eco*RI site in the expected position (data not shown). Three independent pH2-transformed lines contained full-length viral genomes; the predominant form in each, however, did not contain the expected linker *Eco*RI site. Two did contain a minor species corresponding to a viral genome with a linker insertion. After cells were passed for 1 month, DNA was again made from these two lines, and they were found to have lost even this minor species: only full-length proviruses which were insensitive to digestion with *Eco*RI were present. This mutated plasmid apparently has a weak transforming activity, but growth of transformed cells into mass cultures selects for variants which have altered the linker restriction sites. In another mutant, pXB5, a (*Bam*HI)linker had been inserted at exactly the same place as the (*Eco*RI)linker in pH2. pXB was totally transformation defective, presumably because different amino acids are encoded by the two linkers. Thus, this site is clearly within a region important for transformation (Fig. 2).

TABLE 1. Transformation by mutant plasmids[a]

Plasmid	Derivation of plasmid	Linker inserted	Insertion after amino acid no.:	Transformation of NIH/3T3 cells
pH3	pAB160	*Eco*RI	228 of *gag*	+
pR1	pAB160	*Eco*RI	4 of v-*abl*	+
pH4	pAB160	*Eco*RI	15 of v-*abl*	+
pXB8	pABX	*Bam*HI	15 of v-*abl*	+
pH2	pAB160	*Eco*RI	136 of v-*abl*	+[b]
pXB5	pABX	*Bam*HI	136 of v-*abl*	−
pXB4	pABX	*Bam*HI	154 of v-*abl*	−
pXB3	pABX	*Bam*HI	229 of v-*abl*	−
pXB1	pABX	*Bam*HI	268 of v-*abl*	−
pH1	pAB160	*Eco*RI	297 of v-*abl*	−

[a] Linkers were 12 bp in size. The plasmids were cotransfected with M-MuLV DNA. +, Transfection efficiency was comparable to that of the wild type (100 to 500 foci per μg of DNA); −, no foci were observed.

[b] The transfection efficiency for pH2 was lower, ca. 50 foci per μg of DNA.

Mutations abolish tyrosine kinase activity. The only known biochemical activity of the A-MuLV gene product is its tyrosine-specific protein kinase activity. We were interested in determining the effects of the mutations on this activity and whether this correlated with the transforming abilities. The mutants which are defective for transformation, however, gave no phenotype when transfected into NIH/3T3 cells. In one approach to recovering cells that might express the transformation-defective proteins, we cotransfected the mutant plasmids with a construct, pSV2gpt, containing a selectable gene. Although we were able to isolate cell lines containing the mutant genomes, these cell lines were not useful for biochemical analysis of the proteins. We could detect some expression of each of the defective proteins, but their levels were low when compared with transforming proteins in infected cells. In addition, with time in culture the cell lines lost even the low level of defective protein they had synthesized. We attribute these problems to differences in expression and stability of genes in transfected versus infected cells (14). As an alternative approach we chose to

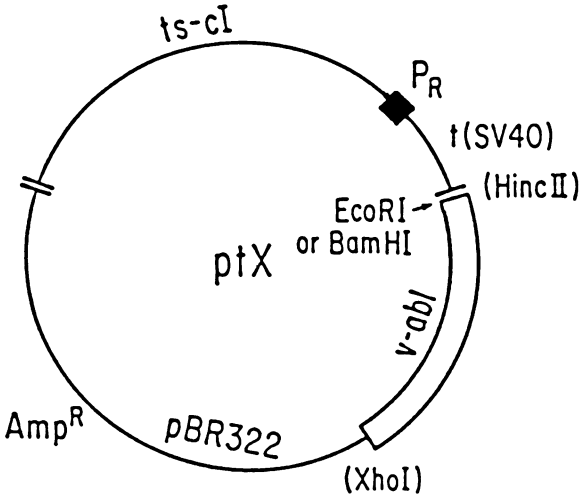

FIG. 3. v-*abl* bacterial expression vector. A map of the bacterial expression plasmid, ptX, is shown. The sites of b-*abl* used for insertion are indicated in parentheses and have been altered. An *Eco*RI site is now where the *Hin*cII site of v-*abl* was (a *Bam*HI site has been placed there in an alternative form of the plasmid used to generate ptH1). The plasmid also contains 58 amino acids of simian virus 40 small t coding sequence fused to v-*abl*. The lambda phage P_R promoter is used and a temperature-sensitive lambda phage *c*I gene (*ts-c*I) is also on the plasmid, making expression from the P_R promoter inducible at a high temperature.

analyze the effects of the mutations on v-*abl* proteins expressed in *E. coli*. Wang et al. (39) have shown that the v-*abl* protein expressed in *E. coli* contains protein-tyrosine kinase activity. The minimum transforming region described above is sufficient to encode this activity (Wang and Baltimore, in press). A mutant protein expressed from v-*abl* coding sequence only up to the *Sac*I site displays no protein-tyrosine kinase activity (Wang and Baltimore, in press) correlating with the inability of the equivalent mutant, pABS, to transform cells.

To determine the effect of our various mutations on the protein-tyrosine kinase activity of the protein made in *E. coli*, we used an expression vector employed in earlier studies (39). It was constructed to use the lambda phage P_R promoter and is heat inducible because of the inclusion in the plasmid of a temperature-sensitive *c*I repressor gene (Fig. 3) (39). To enhance production, v-*abl* is expressed in the construct as a fusion gene with the first 58 amino acids of simian virus 40 small t antigen (Wang and Baltimore, in press). The 5′ 1.2 kb, from *Hin*cII to *Xho*I, of the various mutants of v-*abl* was expressed (see above for details of construction). Each of the mutant clones resulted in the expression of v-*abl*-specific proteins in *E. coli*. To estimate the steady-state level of v-*abl* product, log-phase cells grown at 30°C were shifted to 40°C for 1 h and then labeled for 1 h at 40°C with [^{35}S]methionine. Bacterial cell lysates were then

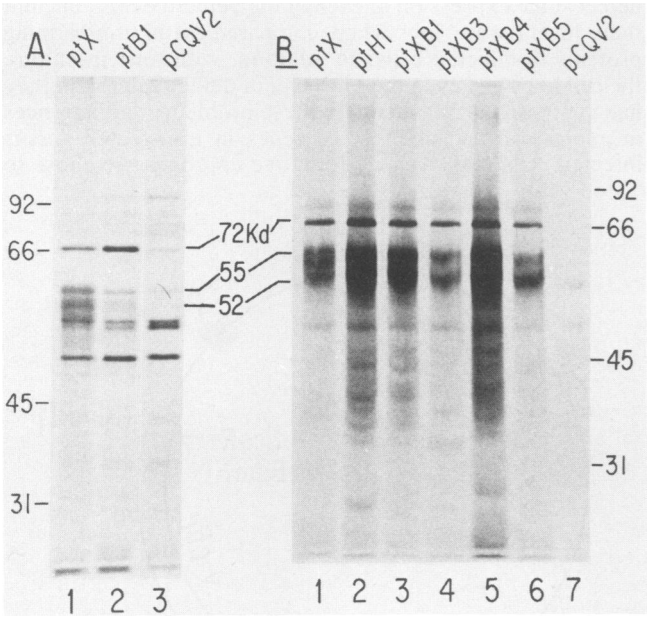

FIG. 4. [^{35}S]methionine labeling of *E. coli*. Bacteria containing the v-*abl* expression vector were labeled for 1 h with [^{35}S]methionine, extracted, immunoprecipitated with anti-AbT serum, and analyzed by SDS–10% polyacrylamide electrophoresis as described in the text. The plasmid present in each sample of labeled bacteria is indicated above each lane. pCQV2 is the parent of ptX but contains no v-*abl* insert. Plasmid ptX contains a wild-type fragment of v-*abl*, whereas the other plasmids contain fragments harboring linker insertions or in the case of ptB1, a deletion of the first 28 amino acids of v-*abl*. These plasmids contain mutations corresponding to those in the viral mutant plasmids; e.g., ptB1 has the same mutation as in pB1. The positions of proteins referred to in the text are indicated in the center of the figure, whereas protein markers of known molecular mass (in kd) are indicated on the outer borders. Panels (A) and (B) represent two separate experiments.

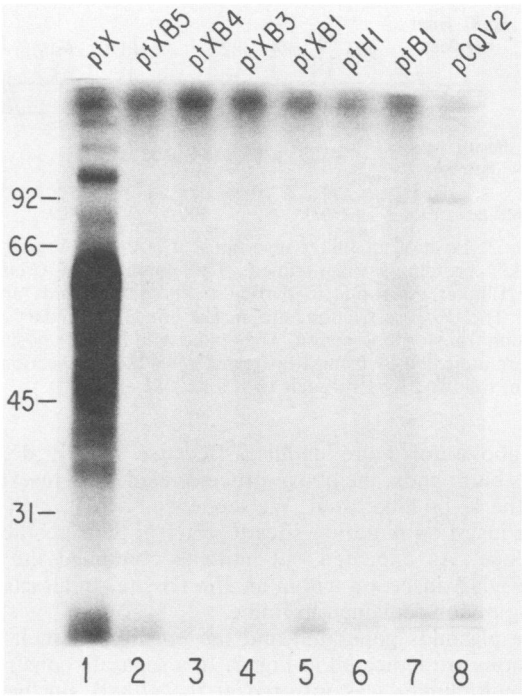

FIG. 5. In vivo $^{32}PO_4^{2-}$ pulse-label of *E. coli*. Bacteria containing the different v-*abl* expression plasmids were induced for 2 h at 40°C and labeled for 15 min with $^{32}P_i$. The cells were extracted and analyzed by SDS–10% polyacrylamide electrophoresis as described in the text. The gel was soaked in 1 N NaOH for 1 h to enrich for the phosphotyrosine-specific signal and developed by autoradiography (7, 8). The plasmid in each bacterial sample is indicated above each lane. The positions of protein markers of known molecular mass (in kd) are marked.

immunoprecipitated with anti-AbT, a serum specific for v-*abl* (43). Bacteria containing only the vector, pCQV2, had some nonspecific proteins that immunoprecipitated (Fig. 4A, lane 3); a plasmid encoding a wild-type v-*abl* protein expressed two specific proteins of 55 and 52 kd (lane 1) (Wang and Baltimore, in press). Also evident was a greatly increased amount of a 72-kd *E. coli* protein (Wang and Baltimore, in press). All of the mutants tested produced easily detectable amounts of *abl*-related proteins (Fig. 4A, lane 2, and Fig. 4B).

To compare the tyrosine-specific kinase activities of the proteins made in *E. coli* at 40°C, we labeled the bacteria with $^{32}PO_4^{2-}$ and examined lysates for alkali-resistant phosphoproteins (39). The wild-type v-*abl* product phosphorylates itself and other bacterial proteins on tyrosine when bacteria are labeled in this manner (39), and as expected, the wild-type plasmid, ptX, caused phosphorylation of a number of bacterial proteins (Fig. 5, lane 1). All but one of the other plasmids were indistinguishable from the negative control, pCQV2, containing no insert (lane 8). Thus, it appears that each of the transformation-defective mutations within the minimum transforming region resulted in a loss of kinase activity as assayed in *E. coli*. There may be some phosphorylation activity in the v-*abl* product of the 5′ deletion mutant, plasmid ptB1 (lane 7), but it too is very strongly reduced. These results demonstrate that the minimum transforming region overlaps strongly with the kinase domain of the protein. Although it is likely that the loss of kinase activity demonstrated here is a reason for the loss of

transforming ability, it is also possible that these proteins are defective for other reasons, such as protein instability or loss of other unknown activities.

DISCUSSION

The mutants described in this paper demonstrate the importance of the 5′ 1.2 kb of v-*abl* sequence for transformation of NIH/3T3 cells by A-MuLV. This minimum transforming region is sufficient for transformation when expressed as a fusion protein with *gag* or with only the first 34 amino acids of *gag* (22). Small 5′ or 3′ deletions into this region, as well as four amino acid insertions, produced transformation-defective viruses. The effect on protein-tyrosine kinase activity of these mutations was assayed, and in each case mutations which caused a loss of transforming activity also caused a loss of kinase activity.

Mutations within the minimum transforming region. The 5′- and 3′-end deletions described here both caused a complete transformation deficiency, indicating that the N-terminal border of the minimum transforming region lies between amino acids 5 and 28, whereas the C-terminal border is between amino acids 356 and 408. The linker insertions demonstrate the requirement for much of the internal sequence of this region. Linker insertions near the 5′ end had no effect, but five of six internal insertions caused a total loss of the ability to induce transformation. The sixth, pH2, was also defective in that it had a lowered transfection efficiency and its (*Eco*RI)linker sequence was lost upon passage of the cells. In addition, a genome with a (*Bam*HI)linker inserted at the same position was totally transformation defective.

Correlation of kinase activity and the minimum transforming region. As a measure of kinase activity, we expressed v-*abl* in *E. coli* and assayed for incorporation of alkali-resistant phosphate into protein. Each of the transformation-defective linker insertion mutants had lost kinase activity by this assay. The N-terminal deletion of 28 amino acids (in plasmid pB1) had a severely reduced activity. Wang and Baltimore found that a C-terminal deletion from the *Sac*I site also caused a loss of tyrosine kinase activity (in press).

The correlation of kinase activity and transformation ability demonstrated here strengthens the belief that the kinase activity is responsible for the transforming activity of the A-MuLV. Such experiments, however, cannot unambiguously demonstrate that kinase activity is necessary for transformation because we cannot be sure that another defect of unknown nature might not accompany the loss of kinase activity. Our inability to recover cells stably expressing the mutant protein even leaves open the possibility that instability of the protein due to intracellular proteases might correlate with loss of kinase activity. Because the linker insertion mutations are small and discrete, we doubt that all of them would induce major changes in the folding of the protein. The appearance of the same two bands of stable v-*abl*-encoded protein in *E. coli* argues that the mutations do not drastically disrupt the structure of the protein because otherwise it would be degraded in *E. coli*. In fact, the stability of the protein encoded by the minimum transforming region is striking—the rest of the v-*abl*-encoded sequence is cleaved off and degraded rapidly in *E. coli* (39; Wang and Baltimore, in press).

Minimum transforming region of three oncogenes encoding a kinase. The studies reported here complement work from other laboratories on mutants of viruses that encode tyrosine-specific protein kinases. In Fig. 6, we show the location of our mutations along with others. For this purpose we have aligned three amino acid sequences, those of v-*abl* (23; R. Lee and D. Baltimore, unpublished data), v-*src* (30, 36), and v-*fps* (31) (the alignment was by L. Shiman and D. Baltimore [unpublished data]). The extensive amino acid homology of these and other protein-tyrosine kinases has been widely noted (3, 23, 31). Certain landmarks are evident, including the tyrosine phosphorylated in vivo on pp60$^{v\text{-}src}$ (30, 36) and the lysine that was affinity labeled by ATP in the cAMP-dependent protein kinase (2, 33) (both marked by asterisks in Fig. 6).

The sequence in Fig. 6 is limited to the region encompassing the v-*abl* minimum transforming region. This was defined previously for v-*abl* by deletions to begin no earlier than amino acid 5 and to end before the *Pst*I site at amino acid 408 (22). These limits can be slightly refined. (For this discussion we will refer to amino acid numbers in the v-*abl* sequence, but the equivalent numbers for v-*src* and v-*fps* can be interpreted from Fig. 6.) Three insertions before amino acid 15 had no effect on transforming efficiency, suggesting that the region up to this position is not required for transformation. The deletion in pB1 abolishes transformation, however, indicating that the minimum transforming region begins between amino acid 15 and 28 in v-*abl*. In v-*fps*, an insertion of three amino acids at amino acid 13 (Tyr) abolishes transformation (35), suggesting that its minimum transforming region may also begin near this position. There is also conserved homology here among the oncogenes, suggesting an important function. For v-*src*, however, a deletion up to amino acid 14 did not ablate transformation of chicken embryo fibroblasts. Deletion to amino acid 31, however, virtually abolished v-*src* transformation (F. Cross and H. Hanafusa, personal communication), suggesting that the minimum transforming region of v-*src* has its 5′ end near that of v-*abl*. Surprisingly, though, deletion of amino acids 34 to 85 in v-*src* gives only a temperature-sensitive transformation defect (5), implying that, at least at the permissive temperature, this region is not needed for transformation.

The 3′ end of the minimum transformation region is probably best defined by examining the amino acid homology of the three proteins. This homology extends to amino acid 377, with the v-*src* and v-*fps* genes ending soon thereafter. A v-*src* deletion from amino acid 366 onward is transformation defective (21). For v-*abl*, some sequence beyond the *Sac*I site at amino acid 356 is needed, but truncation at amino acid 408 (the *Pst*I site) leaves transforming activity. Thus, the data on the mutations are consistent with a need for the entire region of homologous sequence near the 3′ end. In v-*abl*, 631 C-terminal amino acids follow position 377. There is no absolute requirement for this large C-terminal region of the protein, but transformation of lymphoid cells, which may be a more stringent test than transformation of fibroblasts, is more efficient when this region is left intact (22a, 27).

Internal mutations within the v-*abl* minimum transforming region all either abolished or greatly affected transformation. This indicates that much of the region is required, but large segments were not probed. Earlier, the P92 transformation-defective deletion was isolated, which removed the whole central area of the minimum transforming region (11, 42). (We have since found that it arose from a deletion which joined two in-phase 9-bp repeated sequences encoding the amino acids Met-Glu-Arg; see Fig. 6.) Insertion mutants of Ser-Arg-Glu in the place of Tyr residues in the v-*fps* gene have also shown that much of the C-terminal portion of the minimum transforming region of this oncogene must be intact for transformation (35).

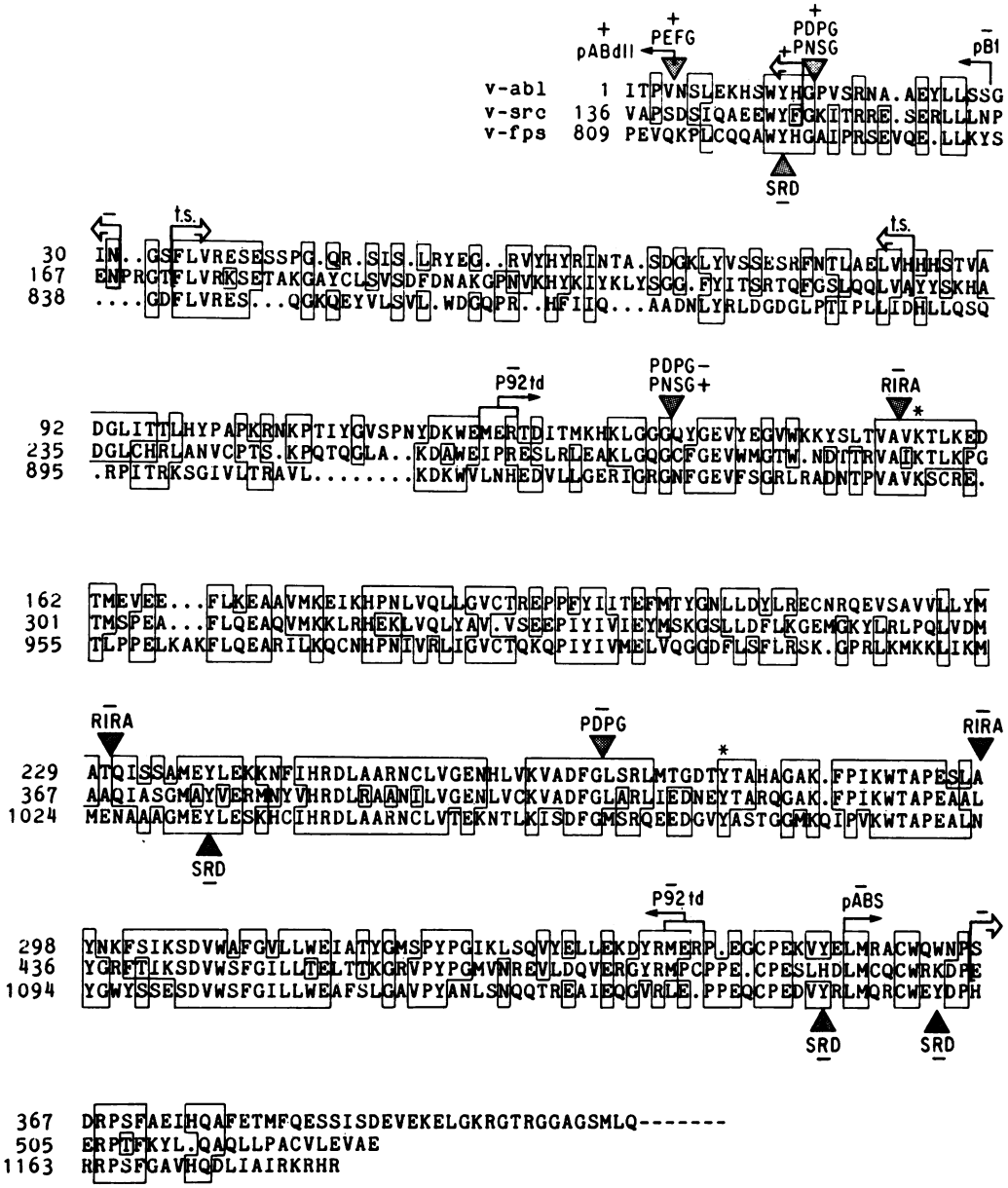

FIG. 6. Position of mutations within the minimum transforming region. The deduced amino acid sequences of sections of v-*abl* (23; Lee and Baltimore, unpublished data), v-*src* (30, 36), and v-*fps* (31) are shown. The amino acid position numbers from the beginning of v-*abl* and v-*src* are indicated to the left of each line. The number for v-*fps* is from the beginning of the *gag-fps* fusion protein, $P130^{fps}$. The C-terminal sequences of v-*src* and v-*fps* are shown, whereas the sequence of v-*abl* continues to amino acid number 1008 (in P160). The sequences were aligned to maximize the homology throughout the *src* family of oncogene tyrosine kinases (Shiman and Baltimore, unpublished data). Similar alignments for *src* and *fps* (31) and *src* and *abl* (23) have been published. Boxes indicate positions in the alignment where the same amino acid is present in two or more of the sequences. The two asterisks indicate (i) the lysine residue whose position aligns with a lysine in the catalytic subunit of the cAMP-dependent protein kinase which can be affinity labeled with an ATP analog and (ii) tyrosine number 416 of $pp60^{v\text{-}src}$, which is phosphorylated in vivo. The position of mutations and the fibroblast-transforming ability of plasmids containing each of the mutations (+ or −) are indicated. In v-*abl*, the mutations are indicated above each line in the appropriate positions. Solid arrows indicate deletions, and the arrows indicate in which direction each sequence is deleted. Triangles indicate the position of linker insertions, with the amino acids that are inserted as a consequence shown above each triangle. Both boundaries of the deletion in $P92td^{abl}$ are shown. Deletions in v-*src* are indicated by open-headed arrows in the appropriate position. Both ends of a deletion which causes a virus temperature sensitive for transformation (5) are also indicated in this way. The positions and transforming abilities of linker insertions in v-*fps* are indicated below each line. These result in the loss of one amino acid (Y) and the insertion of three amino acids (SRD). More details and references are given in the text.

The various existing mutants help to define the region that encodes the protein-tyrosine kinase activity of the oncogene products. For v-*abl*, all nontransforming mutants were kinase negative, except for possible slight residual activity in the N-terminal deletion protein encoded by the pB1 plasmid. For v-*src*, however, the N-terminal segment of the minimum transforming region is not required for kinase activity. This is most dramatically shown by the *ts* deletion of amino acids

34 to 85, which retains kinase activity at both temperatures (5). For v-*src*, at least, the kinase activity appears to involve mainly the C-terminal end of the minimum transforming region. In fact, proteolysis of pp60^{src} produces a kinase-active, C-terminal, 29,000-molecular-weight fragment (4, 18). Also, deletion of amino acids in v-*src* after position 366 abolishes transforming and kinase activities (see Fig. 6; V. Wilkerson and J. T. Parsons, personal communication), as do point mutants at amino acids 292 to 296 (6, 21).

A final suggestion that the C-terminal portion of the minimum transforming region is the kinase domain comes from the properties of antibodies made to different regions of the v-*src* and v-*abl* proteins. For v-*src*, an antipeptide serum against amino acids 360 to 374 neutralizes kinase activity (10). For v-*abl*, antisera to amino acids 138 to 148 and 232 to 355 block kinase activity (16).

These comparisons lead to similar pictures for the v-*abl* and v-*src* minimum transforming regions, except that the N-terminal sequence may be more important to v-*abl* than to v-*src*. A further similarity is that both oncogenes have a requirement for N-terminal amino acids which are not part of the minimum transforming region (9; R. Prywes and D. Baltimore, unpublished data); in A-MuLV they are encoded by *gag*, and in v-*src* they are derived from c-*src*. Because the N-terminal glycines of both proteins contain covalently bound myristate (29; A. Schultz, personal communication), the requirement for the N-terminal sequence might be for specifying myristylation, which could then play a role in the membrane-binding of these proteins.

In spite of the great similarities between v-*abl* and v-*src*, there are crucial biological differences between them. When placed into a common murine retroviral context, v-*abl* efficiently transforms lymphoid cells, as well as established fibroblasts, whereas v-*src* affects only fibroblasts and not lymphoid cells (B. Mathey-Prevot and D. Baltimore, unpublished data). Also, the substrate specificity of the purified v-*abl*-encoded kinase is different from that of the v-*src*-encoded kinase (J. G. Foulkes and D. Baltimore, unpublished data [discussed by J. G. Foulkes, B. Mathey-Prevot, B. C. Guild, R. Prywes, and D. Baltimore *in* J. Feramisco, B. Ozanne, and C. Stiles, ed., *Growth factors and transformation*]). The sequence differences between the two minimum transforming regions therefore cause significant differences in the behavior of the two oncogenes, although we cannot tell at present what residues are responsible for encoding *abl*-specific properties. Perhaps the important difference lies in the N-terminal segment of the minimum transforming region.

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