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Genetics

Isolation of temperature-sensitive tyrosine kinase mutants of v-*abl* oncogene by screening with antibodies for phosphotyrosine

(Abelson murine leukemia virus/bacterial expression plasmid/reversible cell transformation/immunoblotting/*in vitro* mutagenesis)

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ABSTRACT **Temperature-sensitive protein-tyrosine kinase (EC 2.7.1.112) mutants of the oncogene v-*abl* have been obtained by a direct screening of kinase mutants in bacteria. The v-*abl* oncogene was expressed in *Escherichia coli* as a *trpE*/v-*abl* fusion protein from the *trp* promoter. The expression plasmid was mutagenized *in vitro* and then transfected into *E. coli*. Bacteria that produced defective tyrosine kinases were distinguished from those producing wild-type v-*abl* kinases by hybridization with antibodies specific for phosphotyrosine. Two independent mutations that generated temperature-sensitive tyrosine kinases were found to be located in a 12-amino acid region in the tyrosine kinase domain of the v-*abl*-encoded protein. These mutant v-*abl* oncogenes displayed temperature-sensitive transforming activity when expressed in NIH 3T3 cells. Cells transformed by these temperature-sensitive tyrosine kinase mutants could be shifted between the transformed and untransformed states by changing their growth temperature. These results confirmed the crucial role of tyrosine kinase activity in the v-*abl*-mediated oncogenesis.**

Studies of RNA tumor viruses have brought about the identification and isolation of some 30 viral oncogenes (1). At least 14 of these viral oncogenes encode protein-tyrosine kinases (EC 2.7.1.112) that share a homologous protein domain sufficient for the expression of the enzyme activity (2). The v-*abl* oncogene, first isolated from the Abelson murine leukemia virus (Ab-MuLV), is a member of this tyrosine kinase gene family. Ab-MuLV is capable of transforming lymphoid and fibroblastic cells (3, 4). The tyrosine kinase activity of the v-*abl*-encoded protein is essential to the transforming activity of Ab-MuLV (5). Although the importance of the kinase activity in transformation is well documented, the mechanism by which these tyrosine kinases mediate cell transformation is unclear.

A crucial step toward the understanding of the role of protein-tyrosine kinases in cell transformation is to identify the relevant substrate proteins of the kinases. Several proteins have been shown to become tyrosine phosphorylated in cells transformed by the tyrosine kinase oncogenes (6). However, recent evidence indicates that tyrosine phosphorylation of those proteins may not be relevant to the transformation process (7). The v-*abl*-encoded tyrosine kinase has been shown to phosphorylate a variety of substrates, including bacterial proteins (8, 9). The apparent lack of specificity of the tyrosine kinases may account for the difficulty in finding relevant substrates by the direct examination of tyrosine-phosphorylated proteins in transformed cells.

A different approach for defining the important substrates of the v-*abl* tyrosine kinase is to first identify the immediate biological effect that the kinase has on cells. After finding a cellular function that is directly affected by the v-*abl* tyrosine kinase activity, an *in vitro* assay for that function can be set up and used to define the relevant substrates of the kinase. Cells transformed by the v-*abl* oncogene differ from untransformed cells in a wide range of biological processes, including metabolism, membrane transport, and gene expression. Alterations of biological processes that are induced by a tyrosine kinase would be expected to correlate closely with changes in kinase activity. To correlate kinase activity with a biological process requires that the kinase activity be switched on and off *in vivo*. Temperature-sensitive (ts) mutants of the kinase function provide an efficient means of achieving such regulation. Traditionally, ts mutants of an oncogene have been isolated by selecting for ts defects in transformation. Many of the ts transformation-defective mutants of v-*src* have ts tyrosine kinase activity but at least one of these ts mutants (LA32) has normal kinase activity at the nonpermissive temperature, indicating that transformation-defective mutations can affect other aspects of the oncogene required for transformation (10). To select directly for mutations that alter the tyrosine kinase activity, we took a different approach.

It has been shown that the v-*abl*-encoded protein is an active tyrosine kinase in *Escherichia coli* (9, 11). Normal *E. coli* contain no detectable phosphotyrosine (*P*-Tyr) but when the v-*abl* protein is expressed the *P*-Tyr level becomes high (9). Using a high-affinity anti-*P*-Tyr antibody that one of us has isolated (12), it is easy to distinguish between *E. coli* colonies with or without *P*-Tyr. Our strategy for obtaining ts tyrosine kinase mutants is to mutagenize the v-*abl* expression plasmid DNA *in vitro* and then to screen bacterial colonies containing mutant plasmids with the anti-*P*-Tyr antibody. By counter screening with a monoclonal anti-*abl* antibody (which was prepared by Hanna Kupfer), the kinase mutants can be distinguished from mutants not expressing any v-*abl* protein. This strategy has allowed the isolation of two ts tyrosine kinase mutants of v-*abl*. These ts kinase mutants exhibited ts transforming activity when they were subsequently expressed in mammalian cells.

MATERIALS AND METHODS

Plasmids. Expression of the v-*abl* sequence from *Hin*cII to *Hin*dIII sites, 3638 base pairs (bp) containing the entire v-*abl* coding sequence except the first 4 amino acids (9), was achieved using the *trp* promoter of *E. coli* (13). The v-*abl* coding sequence was fused with the first 1388 bp of *trpE* protein and a *Bam*HI site was placed at the *trpE*/v-*abl* junction (Fig. 1). This expression plasmid, pCP4, produced a M_r 92,000 *trpE*/v-*abl* protein with tyrosine kinase activity. Plasmid pAB/pCP4 was constructed by replacing the *Bst*EII–*Xho* I fragment of cloned Ab-MuLV P160 DNA, pAB160 (14), with the *Bam*HI–*Xho* I fragment of pCP4 (Fig.

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Abbreviations: ts, temperature sensitive; *P*-Tyr, phosphotyrosine; Ab-MuLV, Abelson murine leukemia virus.

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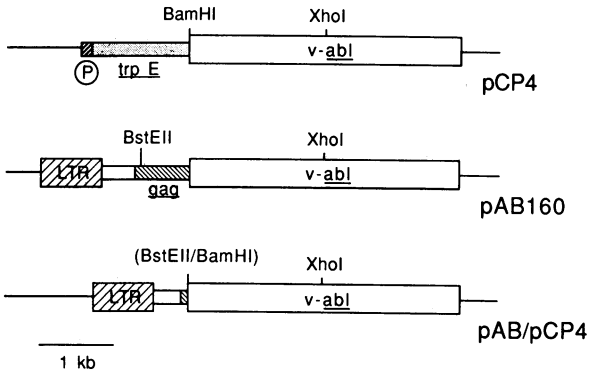

FIG. 1. Bacterial and retroviral expression plasmids. Plasmid pCP4 expresses a *trpE*/v-*abl* fusion protein from the tryptophan promoter of *E. coli*. Plasmid pAB160 is a cloned Ab-MuLV genome (14). Plasmid pAB/pCP4 was constructed by replacing the *Bst*EII–*Xho* I fragment of pAB/160 with the *Bam*HI–*Xho* I fragment of pCP4. kb, Kilobase; LTR, long terminal repeat; P, promoter.

1). This plasmid produces a protein containing only 34 amino acids of Moloney murine leukemia virus *gag*-encoded protein fused with the v-*abl* protein. Plasmid pAB/pCP4 transforms 3T3 fibroblasts. Mutant plasmids pAB/DP and pAB/RK were constructed similarly to pAB/pCP4, starting with the mutant plasmids pDP and pRK.

Mutagenesis. Two types of insertional mutagenesis were used. In the first method, pCP4 was linearized with *Dde* I (12 of a total of 38 sites in the v-*abl* tyrosine kinase domain), the three-base overhang was filled in with the Klenow fragment of *E. coli* polymerase I, and the full-length linear plasmid was purified and recircularized with T4 DNA ligase. In the second method, 6-bp *Eco*RI or *Kpn* I linkers were ligated onto pCP4 linearized with *Hae* III (13 of 58 sites in the v-*abl* tyrosine kinase domain), *Rsa* I (8 of 19 sites), or *Alu* I (10 of 47 sites), all of which generated blunt ends. After digestion of the linkers, full-length linear plasmid was ligated with T4 DNA ligase.

Screening Bacterial Colonies with Antibodies. Mutagenized plasmids were transfected into *E. coli* strain C600 and ampicillin-resistant colonies were grown on Millipore HATF nitrocellulose at 40°C. Two replica filters were made of the master filter, and the bacterial colonies on the replica were lysed in chloroform vapor and incubated with antibodies as described by Helfman and coworkers (15). In the *Dde* I mutagenesis, both replicas were grown at 40°C, one filter was incubated with affinity-purified antibody for *P*-Tyr (0.3 μg/ml), and the other filter was incubated with a monoclonal anti-*abl* antibody (1 μg/ml). One hundred and eighty tyrosine kinase mutants—i.e., those that scored positive with the anti-*abl* antibody and negative with the anti-*P*-Tyr antibody—were picked from the master filter and rescreened with anti-*P*-Tyr antibody at 32°C and 40°C to find ts kinase mutants. Plasmids mutagenized by linker insertions were screened directly for ts kinase mutants. The two replicas were grown at 32°C or 40°C and both filters were hybridized with anti-*P*-Tyr antibodies. ts Mutants were those that scored positive at 32°C but negative at 40°C (see Fig. 2*A*). These colonies were picked and rescreened at 40°C with the anti-*abl* antibody to distinguish kinase mutants from protein expression mutants.

Immunoblotting. Preparation of bacterial extracts, NaDodSO$_4$ gel electrophoresis, electrophoretic transfer of protein to nitrocellulose, and immunoblotting with anti-*P*-Tyr and anti-*abl* antibodies were performed as described (12). Quantitation of kinase activity was accomplished by slot blotting bacterial extracts onto nitrocellulose. Extracts were diluted in saline (50 mM Tris·HCl, pH 7.5/150 mM NaCl) and 20% methanol; after passing through a slot blot apparatus (Schleicher & Schuell), samples were washed with 20% methanol/10% acetic acid and then hybridized with antibodies as described (12). Quantitation was achieved by densitometric scanning of autoradiographs of the nitrocellulose filters. To quantitate the level of antigen, the signal was compared to a standard curve of dilutions of a purified v-*abl* protein produced in bacteria from the expression plasmid ptabl130 (11).

***In Vitro* Tyrosine Kinase Activity.** The procedure for the preparation of cell-free extracts from bacteria and measurement of angiotensin II and casein kinase activity were as described by Wang and Baltimore (11). Cells were sonicated in 20 mM Tris·HCl; pH 8.0/2 mM EDTA/100 mM NaCl/10 mM 2-mercaptoethanol at 4°C. The sonicate was centrifuged at 45,000 × *g* for 30 min to remove cell debris and membranes. Angiotensin II or casein kinase activity was measured at 23°C, 30°C, or 40°C. Wild-type extracts showed activity at all three temperatures (150 pmol/min per mg, 30°C: angiotensin II assay), whereas mutant extracts showed no detectable activities with angiotensin II or casein as substrates.

Transfection/Cell Culture. Transfection of Ab-MuLV P160 plasmid, pAB160, and its derivatives into NIH 3T3 cells was as described by Goff and coworkers (16) using the calcium phosphate method (17). Clone seven of NIH 3T3 cells (from Geoffrey M. Cooper) was used in these studies, and cells were cultured on Dulbecco's modified Eagle's medium with 10% fetal calf serum (Flow Laboratories). After transfection, cells were maintained at 32°C for 3 days and then were split in half and cultured at 32°C or 39°C. Foci were counted after 2 weeks.

RESULTS

Isolation of ts Tyrosine Kinase Mutants. The v-*abl* expression vector pCP4 (Fig. 1) was mutagenized by insertional mutagenesis and then screened in *E. coli* with anti-*P*-Tyr antibodies as described in *Materials and Methods*. In five different linker mutageneses (*Kpn* I linker into *Hae*III, *Rsa* I, or *Alu* I sites and *Eco*RI linker into *Rsa* I or *Alu* I sites), between 400 and 4500 colonies were screened; only one ts mutant was obtained from the insertion of *Kpn* I linker into an *Rsa* I site. Detection of this mutant in the primary screen is shown in Fig. 2*A*. Approximately 3000 colonies were screened in the *Dde* I fill-in mutagenesis and four ts mutants were picked. These mutants are true ts kinase mutants because they produce v-*abl* proteins of the correct molecular weight at 32°C and 40°C but the tyrosine phosphorylation of these proteins is greatly reduced at 40°C (Fig. 2*B*).

Localization of the mutations in the v-*abl* sequence was achieved by restriction mapping of the mutant plasmid DNA. Digestion of the *Dde* I-mutagenized plasmids with *Dde* I showed that all four mutants were missing the same *Dde* I site, which is located at base pair 633 of the v-*abl* sequence. This was then confirmed by sequencing the mutant DNA across the site of the mutation. The addition of 3 bp at that position of the v-*abl* sequence introduces a methionine into the v-*abl* protein without altering any other amino acids (Fig. 3). This *Dde* I mutation is referred to as DP. Digestion of the *Kpn* I linker insertion mutant showed that the 6-bp *Kpn* I linker is added to the *Rsa* I site located at base pair 669 of the v-*abl* sequence and it introduces two amino acids, valine and proline, into the v-*abl* protein without changing flanking amino acids (Fig. 3). This *Kpn* I mutation is referred to as RK. It is interesting to find that the two ts kinase mutations are located in the same region within the tyrosine kinase domain of the v-*abl* protein.

Effect of Temperature on Mutant Kinase Activity. The wild-type *trpE*/v-*abl* protein produced from pCP4 can phosphorylate exogenous substrates such as angiotensin II or

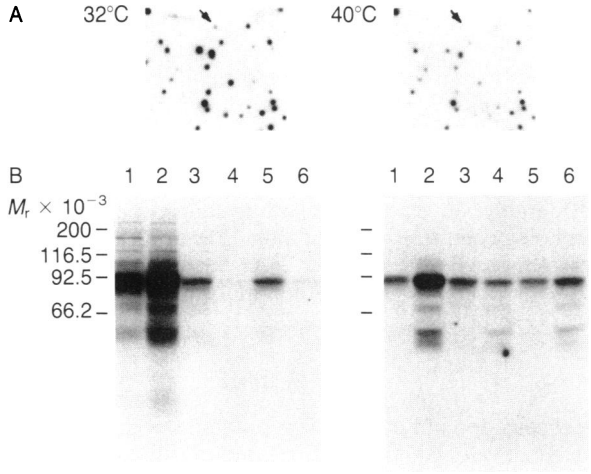

FIG. 2. Isolation and expression of the ts mutants. (*A*) Sections of primary screen with anti-*P*-Tyr antibody. Bacteria were transfected with *Rsa* I–*Kpn* I linker mutagenized DNA and grown at 32°C and 40°C. Hybridization of bacterial colonies on nitrocellulose filters with anti-*P*-Tyr antibodies is described in the text. The arrows point to the location of a bacterial colony expressing the mutant RK. (*B*) Immunoblots of extracts prepared from bacteria expressing pCP4 (lanes 1 and 2), pDP (lanes 3 and 4), and pRK (lanes 5 and 6) plasmids and that were grown at either 32°C (lanes 1, 3, and 5) or at 40°C (lanes 2, 4, and 6). The lysates of ≈4 × 10^6 bacteria were loaded per lane of a 7% $NaDodSO_4$/polyacrylamide gel. Hybridizations were with either anti-*P*-Tyr (*Left*) or anti-*abl* (*Right*) antibodies.

casein on tyrosines. Its activity can be demonstrated *in vitro* using bacterial extract as a source of the enzyme (see *Materials and Methods*). Although the kinase activity of the mutant proteins can be observed *in vivo* (Fig. 2*B*), their activity could not be measured *in vitro*. Extracts prepared from 32°C cultures of bacteria expressing the pDP or pRK proteins failed to phosphorylate angiotensin II or casein

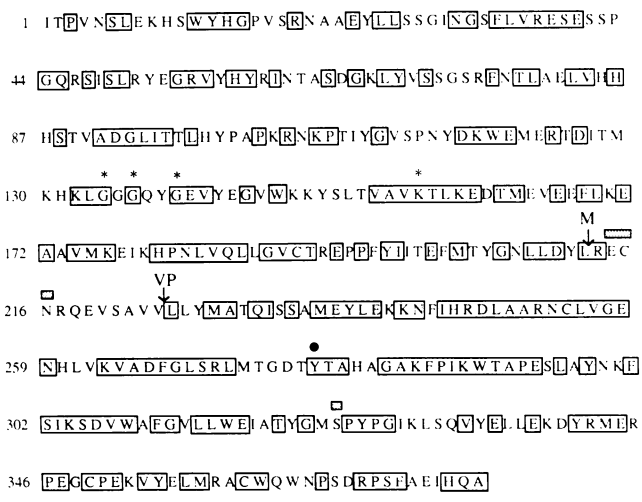

FIG. 3. Location of DP and RK mutations. The sites of the mutations are denoted by arrows, indicating the inserted amino acids: a methionine inserted between amino acids 212 and 213 (DP) and valine/proline inserted between amino acids 224 and 225 (RK). Amino acids that share homology with v-*src* or v-*fps* are boxed. Alignment is as described by Prywes and coworkers (5). The amino acid signatures of the ATP binding domain are denoted by asterisks (18, 19). The major tyrosine autophosphorylation site of v-*abl in vivo* (20) that corresponds to tyrosine 416 of v-*src* is denoted by a dot. The sites corresponding to the v-*src* sites of the mutations that generate the ts mutant tsNY68 are denoted by a shaded bar indicating (*i*) a deletion of three amino acids (Gly-Glu-Met) and (*ii*) a change altering valine to methionine (21).

despite the fact that these extracts contained intact mutant proteins that were tyrosine phosphorylated (data not shown). It is possible that the mutant proteins were less stable and they were easily denatured during the preparation of the cell-free extracts or that the mutations affected the ability of these two v-*abl* proteins to phosphorylate nonspecific substrates such as angiotensin II or casein.

To quantitate the effect of temperature on the mutant kinase activity in bacteria, steady-state levels of *P*-Tyr and v-*abl* protein were determined by immunoblotting with anti-*P*-Tyr and anti-*abl* antibodies. A purified v-*abl* protein—*tabl*130, which is stoichiometrically tyrosine phosphorylated—was used as a standard for the anti-*P*-Tyr and the anti-*abl* antibody hybridizations (see *Materials and Methods*). Because there is no dephosphorylation of *P*-Tyr in bacteria, the ratio of the amount of *P*-Tyr to the amount of v-*abl* protein present in the bacterial extracts represents the apparent tyrosine kinase activity *in vivo*. The major tyrosine-phosphorylated protein in the bacterial cells is the v-*abl* protein (Fig. 2*B*), so the *P*-Tyr/*abl* ratio reflects the autophosphorylation activity of the v-*abl* protein. In several determinations, the *P*-Tyr/*abl* ratio at 32°C was approximately equivalent for the two mutants. The wild-type pCP4 had a 2.5-fold higher *P*-Tyr/*abl* ratio than the mutants at 32°C. The wild-type *trpE*/v-*abl* protein appeared to be slightly ts; its *P*-Tyr/*abl* ratio at 40°C was ≈60% of that at 32°C. In both mutant bacteria, the *P*-Tyr/*abl* ratios decreased significantly with increase in temperature: the *P*-Tyr/*abl* ratio of DP at 40°C was lower by a factor of 11 than at 32°C and the RK *P*-Tyr/*abl* ratio dropped by a factor of 13 at 40°C.

Transforming Activity of the ts Kinase Mutants. The two ts kinase mutants were placed into cloned Ab-MuLV DNA. The resulting plasmids, pAB/RK and pAB/DP, contained the full-length Ab-MuLV genome minus 601 bp of Ab-MuLV *gag* sequence, as did the analogously constructed pAB/pCP4 (Fig. 1). These plasmids were transfected into NIH 3T3 cells. To determine their transforming efficiency with respect to temperature, a transfected plate of cells was divided equally and then grown at 32°C and either 37°C or 39°C. Ab-MuLV pAB160 and pAB/pCP4 gave similar numbers of foci at either temperature, showing that transformation induced by wild-type v-*abl* oncogene is not affected by growth temperature (Table 1). The ts mutant Ab-MuLVs, however, displayed temperature sensitivity in focus-forming ability (Table 1). Mutant pAB/DP gave no foci at 39°C in four independent experiments. It produced low numbers of foci at 37°C. Mutant pAB/RK was not as tight as pAB/DP at 39°C, and, again, more foci were produced at 37°C. At 32°C, these two mutants had transforming activity comparable to pAB160 but they were less efficient than their wild-type counterpart pAB/pCP4. The lower transforming activity might be due to a lower kinase activity of these two mutants as compared to pAB/pCP4 at 32°C.

Three independent foci for each mutant were isolated and were tested for their ability to form colonies in soft agar. Isolated cells transformed by all four types of Ab-MuLV could form colonies in soft agarose (Table 1). Again, the ts kinase mutant cells grew efficiently in soft agarose at 32°C but not at 39°C. These results further confirmed that the ts kinase v-*abl* mutants had ts transforming activity.

The ts transforming activity of the DP and RK mutant isolates could be correlated with their ts kinase activities in NIH 3T3 cells. Immunoblotting with anti-*P*-Tyr antibodies showed reduction of tyrosine-phosphorylated proteins in pAB/DP or pAB/RK transformed cells at 39°C (Fig. 4*A*). Part of this reduction could be due to a lower steady-state level of the Ab-MuLV proteins at 39°C, as revealed by immunoblotting the same lysates with anti-*abl* monoclonal antibody (Fig. 4*B*). The wild-type pAB/pCP4 protein level decreased by 25% and the mutant protein levels decreased by 50% at 39°C, relative to 32°C levels. The *P*-Tyr/*abl* ratio of

Table 1. Foci formation and colony formation of the ts mutants

DNA	Foci formation,* foci per μg of DNA				Soft agar colony formation,† colonies per 1000 cells seeded	
	32°C	39°C	32°C	37°C	32°C	37°C
pAB160	605	1027	842	1000	238	245
pAB/pCP4	2356	2032	1344	1932	456	477
pAB/DP	732	0	132	2	138	0
pAB/RK	656	12	514	36	410	6
None‡	0	0	0	0	0	0

*One microgram of retroviral plasmids was cotransfected with 10 μg of helper virus pZAP, a cloned Moloney murine leukemia virus (22). Transfected plates were grown for 3 days at 32°C and then were split into two plates, which were grown at 32°C and 37°C or 32°C and 39°C. The results of two experiments are reported. Foci were counted after 2–3 weeks.

†Colony formation was assayed with cells from isolated foci, which were seeded at 1000 cells per plate. Colonies were scored after 3–4 weeks at both temperatures. Three independent isolates were assayed for each mutant, which all expressed similar ts behavior. The results of one representative isolate for each mutant are presented above.

‡pZAP alone was transfected to test for foci formation; NIH 3T3 fibroblasts were used for colony formation assay.

pAB/pCP4 was not affected by temperature, whereas the *P*-Tyr/*abl* ratio of the two mutant proteins decreased by a factor of 2–3 at 39°C (Fig. 4). The reduction of mutant *P*-Tyr/*abl* ratio at 39°C in 3T3 cells was smaller than that in bacteria. Because tyrosine phosphatases are present in 3T3 cells, the *P*-Tyr/*abl* ratio does not represent the mutant protein-tyrosine kinase activity. The *P*-Tyr level of several other proteins, most notably one of M_r 76,000, also decreased in pAB/DP and pAB/RK transformed cells at 39°C (Fig. 4*A*). These proteins did not react with anti-*abl* antibody, indicating that they might be endogenous substrates for the v-*abl* kinase.

The transformed phenotype of cells expressing the ts kinase mutants could be reversed by shifting the growth temperature. At 32°C all of the isolates expressing ts mutant proteins had a refractile, rounded morphology similar to those cells expressing the wild-type pAB160 or pAB/pCP4 proteins (Fig. 5). One hour after shifting to 39°C or 37°C, the pAB/DP or pAB/RK transformed cells began to revert back to the normal flattened morphology (Fig. 5). After 6–8 hr at 37°C or 39°C, all of the ts cell lines resembled untransformed 3T3 cells, which are flat, nonrefractile, and contact inhibited (Fig. 5). Shifting growth temperature from 39°C to 32°C induced transformed phenotype in the isolated cell lines expressing the ts mutants. The process of transformation was considerably slower than the reversion process. Fusiform morphology could be detected after 11 hr at 32°C for cell lines expressing pAB/RK and after 24 hr for cell lines expressing pAB/DP. Complete transformed morphology was obtained after 24–48 hr at 32°C.

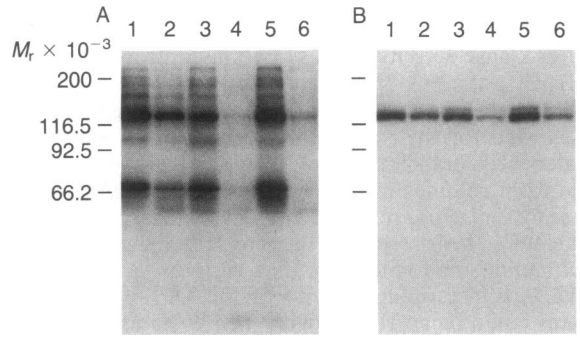

FIG. 4. ts Tyrosine phosphorylation in mutant Ab-MuLV-transformed cells. Extracts were prepared from isolates of foci from cells transformed by pAB/pCP4 (lanes 1 and 2), pAB/DP (lanes 3 and 4), and pAB/RK (lanes 5 and 6), according to the method described by Wang (12). These cells were grown at either 32°C (lanes 1, 3, and 5) or at 40°C (lanes 2, 4, and 6) for 48 hr. The lysates of 1×10^5 cells were loaded per lane of a 7% $NaDodSO_4$/polyacrylamide gel. Hybridizations were with either anti-*P*-Tyr (*A*) or anti-*abl* (*B*) antibodies.

DISCUSSION

Direct selection of tyrosine kinase mutants with the methods we have developed is an efficient way of isolating ts mutants because a large number of mutants can be screened with minimal efforts. This method is made possible by three factors: (*i*) the isolation of high-affinity anti-*P*-Tyr antibody (12), (*ii*) the lack of endogenous tyrosine kinases in bacteria (9), and (*iii*) the ability of the v-*abl* protein kinase to function in *E. coli* (9). This method can be applied to isolate mutants of other tyrosine kinases if the enzyme retains its activity in bacterial cells. Characterization of mutants in the different tyrosine kinases of this gene family will facilitate the elucidation of the structural and functional relationship of these homologous proteins.

The mutagenesis performed in this investigation introduced into the v-*abl* sequence mutations at a total of 93 sites, of which 43 are in the tyrosine kinase domain (11). Of the 43 sites, 31 were mutated by the insertion of two different linkers. Three thousand colonies from the *Dde* I mutagenesis were screened with anti-*P*-Tyr and anti-*abl* antibodies, where 15% of the *abl*-positive colonies were found to be *P*-Tyr-negative. There are a total of 39 *Dde* I sites in plasmid pCP4, of which 12 are in the v-*abl* tyrosine kinase domain. The 15% kinase mutant frequency showed that the majority of the *Dde* I insertions in the kinase domain gave rise to kinase mutants. The four DP mutants were picked from 180 colonies that exhibited mutant phenotype in the primary screen. The repeated isolation of the same DP mutation indicates that they most likely do not contain secondary mutations. The RK mutant was obtained by directly screening 900 colonies at two temperatures with anti-*P*-Tyr antibodies. The high-frequency occurrence of these ts mutants (2 of 43 sites mutated) indicates that insertional mutagenesis may be well suited for obtaining conditional mutants of tyrosine kinase proteins.

It is interesting to find that the two independent mutations, addition of methionine between v-*abl* amino acids 212 and 213 and addition of valine/proline between amino acids 224 and 225, are located only 12 amino acids apart in the v-*abl* tyrosine kinase domain. The mutations are not located at the ATP binding site or the autophosphorylation site, which are found in all of the homologous tyrosine kinases (Fig. 3). Recently, Nishizawa and coworkers (21) have cloned the genome of a ts transformation-defective v-*src* mutant, tsNY68. They found that two mutations are required to produce the ts phenotype: a deletion of 3 amino acids and a substitution mutation (Fig. 3) (21). Interestingly, the 3-amino acid deletion (amino acids 315–317) of tsNY68 is in a v-*src* 12-amino acid region comparable to that defined by the two ts v-*abl* mutants (Fig. 3). The fact that both ts v-*abl* mutants and one ts v-*src* mutant have alterations flanking or within this 12-amino acid region indicates that it might be important for maintaining the structural integrity of the tyrosine kinase domain.

The 12 amino acids flanked by the two ts mutants DP and RK are in fact the largest nonconserved region found in all the

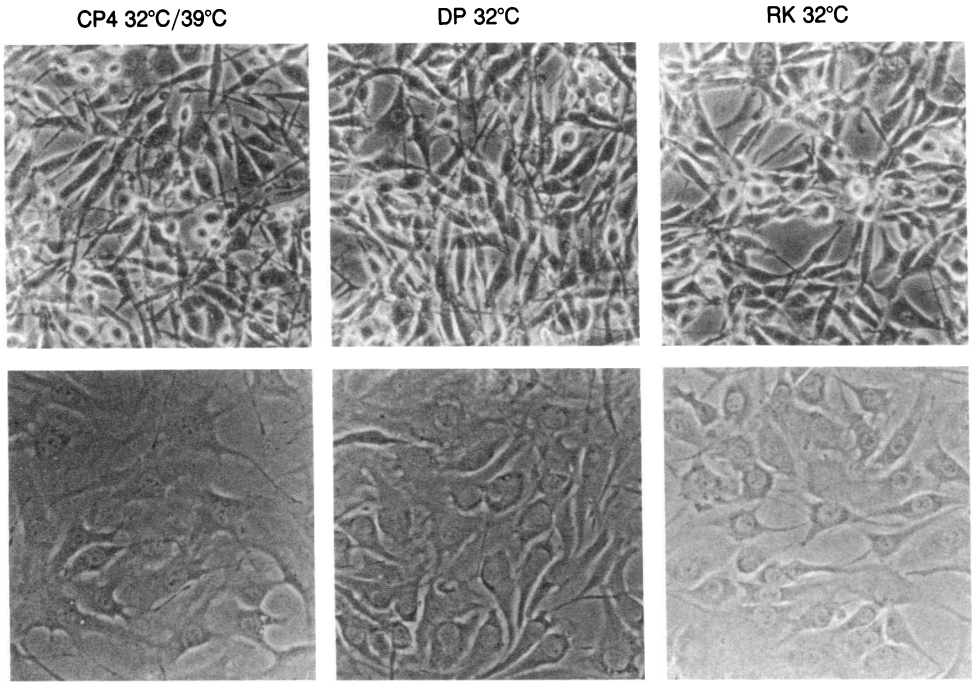

FIG. 5. Morphology of cells expressing wild-type or mutant v-*abl* at two temperatures. Cells isolated from foci produced by transfection with pAB/RK or pAB/DP were grown at 32°C or 39°C for 3 days. Cells transformed by wild-type pAB/pCP4 or untransformed NIH 3T3 cells have identical morphologies at 32°C and 39°C. (×160.)

otherwise homologous tyrosine kinases. This nonconserved region contains from 11 to 17 amino acids in the v-*abl*-, v-*src*-, v-*fes*/*fps*-, v-*fgr*-, v-*yes*-, v-*ros*-, and v-*erbB*-encoded tyrosine kinases and from 79 to 109 amino acids in the v-*fms*, v-*kit*, and platelet-derived growth factor receptor tyrosine kinases (2, 23–25). Because this region contains unique sequences for each tyrosine kinase, it might be involved in conferring specificity to the function of the otherwise similar tyrosine kinases—e.g., the nonconserved region might dictate substrate specificity. DP and RK ts mutants are capable of autophosphorylation, which was shown to be ts in bacteria and in mammalian cells. Although the ts mutants appeared to be able to phosphorylate endogenous substrates (e.g., M_r 76,000 protein in 3T3 cells), the mutant kinases could not phosphorylate exogenous substrates such as angiotensin II or casein *in vitro* even at the permissive temperature. It is possible that the mutations greatly reduced the stability of the proteins, so they were easily denatured *in vitro*, or that the mutations altered the substrate specificity of the tyrosine kinases, so that they were no longer able to phosphorylate the nonspecific substrates.

Demonstration that ts tyrosine kinase mutants of v-*abl* are ts for transformation proves that the kinase activity is crucial to the transforming function. The v-*abl* tyrosine kinase activity is required to maintain the transformed phenotype because cells transformed by the ts kinase mutants reverted back to normal morphology shortly after a shift to the nonpermissive temperature. These ts tyrosine kinase mutants provide the means to regulate the v-*abl* kinase activity *in vivo*, which will facilitate the analyses of the biological function of the v-*abl* tyrosine kinase in cells.

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