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Temperature-Sensitive Mutants of Abelson Murine Leukemia Virus Deficient in Protein Tyrosine Kinase Activity

ALAN ENGELMAN AND NAOMI ROSENBERG*

Departments of Molecular Biology and Microbiology and Pathology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111

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The effect of two missense mutations in *abl* on transformation by Abelson murine leukemia virus was evaluated. These mutations led to the substitution of a histidine for Tyr-590 and a glycine for Lys-536. Both changes gave rise to strains that were temperature dependent for transformation of both NIH 3T3 cells and lymphoid cells when expressed in the context of a truncated Abelson protein. In the context of the prototype P120 v-*abl* protein, the Gly-536 substitution generated a host range mutant that induced conditional transformation in lymphoid cells but had only a subtle effect on NIH 3T3 cells. The combination of both substitutions gave rise to a P120 strain that was temperature sensitive for both NIH 3T3 and lymphoid cell transformation. The Abelson proteins encoded by the temperature-sensitive strains displayed in vitro kinase activities that were reduced when compared with those of wild-type proteins. In vivo, levels of phosphotyrosine were reduced only at the restrictive temperature. Analysis of cells expressing either the wild-type P160 v-*abl* protein or the P210 *bcr/abl* protein and an Abelson protein encoded by a temperature-sensitive strain failed to correct this defect, suggesting either that tyrosine phosphorylation in vivo is an intramolecular reaction or that the protein encoded by the temperature-sensitive strain is a poor substrate for tyrosine phosphorylation in vivo. These results raise the possibility that tyrosine phosphorylation of Abelson protein plays a role in transformation.

Abelson murine leukemia virus (Ab-MLV) is a replication-defective, highly oncogenic retrovirus that arose via a recombination event between Moloney murine leukemia virus and the proto-oncogene c-*abl* (10). The virus transforms a unique spectrum of cells in vitro, including several fibroblast cell lines and pre-B lymphoid cells (35, 39). In vivo, the virus most commonly induces an acute pre-B-cell lymphosarcoma (33). Ab-MLV encodes one protein containing amino-terminal Moloney murine leukemia virus-derived *gag* determinants fused to v-*abl*-encoded determinants (29, 32, 53, 62). This molecule is a protein tyrosine kinase (59), and cells transformed by Ab-MLV contain elevated levels of phosphotyrosine (44). Consistent with the central role of tyrosine phosphorylation in the transformation process, nonconditional mutants that lack kinase activity fail to transform any cell type (27, 30, 31, 60) and conditional mutants compromised in kinase activity at the restrictive temperature fail to maintain the transformed phenotype of the infected cells (7, 17).

A large number of transforming strains of Ab-MLV, designated by the estimated size of the Abelson protein they encode, have been described (13, 25, 36). All of these strains encode proteins that share *gag*-derived amino acids and v-*abl*-encoded amino acids 237 to 613 (Fig. 1). These latter residues encompass the catalytic and the *src* homologous 2 domains of the molecule, regions present in all cytoplasmic protein tyrosine kinases (11, 37). Unlike these other proteins, c-*abl* and the original wild-type v-*abl*-encoded proteins contain a long carboxyl-terminal region of 631 additional amino acids (11). The role of this region of the protein is not fully understood. However, loss of portions of this region of v-*abl* proteins affects lethality and lymphoid cell transformation (25, 36, 56). In c-*abl* proteins, this region appears to play a role in nuclear localization (54).

Abelson protein and pp60^{src}, the transforming protein of Rous sarcoma virus, have 49% identity within the catalytic domain (11, 29). We previously exploited this feature to construct a temperature-sensitive (*ts*) Ab-MLV strain (7). That mutation leads to the substitution of a histidine for a tyrosine at position 590 of the Abelson protein (Fig. 1) and renders the Ab-MLV P70 strain but not the P160 strain *ts* for transformation (7). The P160 and P70 proteins differ only in the carboxyl-terminal region; P160 contains the entire 631-amino-acid carboxyl-terminal region, whereas P70 contains only the *src* homologous 2 and catalytic domains (7). Thus, the effect of the histidine substitution is masked by the carboxyl-terminal domain of the Abelson protein. A second mutation resulting in the substitution of a glycine for Lys-536 was not tested in virus, but an Abelson protein containing this change displayed reduced tyrosine phosphorylation in *Escherichia coli* at a high temperature (7).

In this report, we examine the effects of the two missense mutations singly and in combination in a variety of Ab-MLV strains that encode Abelson proteins differing in the carboxyl-terminal amino acids. These studies demonstrate that as few as 64 carboxyl-terminal v-*abl* amino acids can mask the effect of the His-590 substitution. Further examination of the different Abelson proteins revealed that the in vitro protein kinase activity of these molecules was of prognostic value in identifying *ts* strains. In vivo, decreases in cellular phosphotyrosine were observed in cells transformed by the *ts* strains at the nonpermissive temperature. Abelson proteins encoded by these strains also displayed subtle decreases in phosphotyrosine at the nonpermissive temperature. Because this defect could not be corrected by expression of an active *abl* protein in the same cell, tyrosine

* Corresponding author.

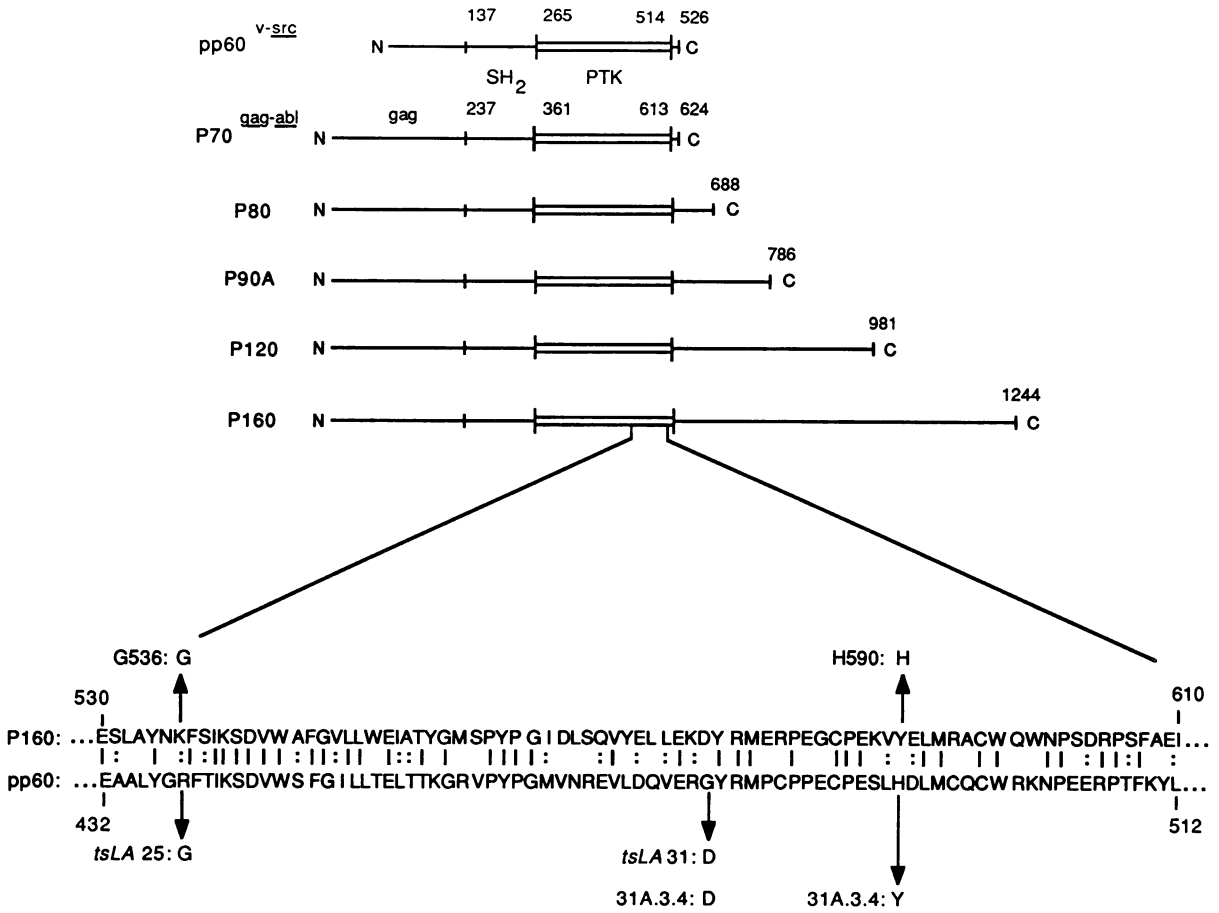

FIG. 1. Abelson protein structure and substitutions leading to temperature-dependent transformation. The upper diagram schematically represents the predicted amino acid sequences of pp60$^{v\text{-}src}$ and the P160, P120, P90A, P80, and P70 Abelson proteins. The sequences (13, 29, 53) were manually aligned for maximum homology; the numbers designate amino acid positions; the open box denotes the catalytic or protein tyrosine kinase domain of the proteins (11); SH_2 denotes the *src* homologous region 2 (37). The lower diagram shows the amino acid substitutions responsible for the conditional transformation displayed by the Rous sarcoma virus mutants *tsLA*25 and *tsLA*31, the substitutions found in the revertant 31A.3.4 (8, 9), and the corresponding substitutions that were evaluated in Abelson proteins. In this part of the diagram, vertical lines denote identity between amino acids and colons denote conservative substitutions. Conservative substitutions were calculated based on the method of Schwartz and Dayhoff (41).

phosphorylation of Abelson proteins may be important for transformation.

MATERIALS AND METHODS

Cell culture and transformation assays. Normal and Ab-MLV-transformed NIH 3T3 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented to contain 10% newborn calf serum (Sigma Chemical Co., St. Louis, Mo.). Transformed lymphoid cells were grown in RPMI 1640 supplemented to contain 10% fetal calf serum (Hyclone Labs, Inc., Logan, Utah) and 50 μM 2-mercaptoethanol. Ab-MLV stocks were prepared from transformed NIH 3T3 cells, and the titer of focus-forming virus was determined by using the NIH 3T3 transformation assay (39). The retroviral construct expressing *bcr/abl* cDNA, JW-RX (24), was a gift of J. McLaughlin, University of California, Los Angeles. In vitro bone marrow transformation assays were performed as described previously (34).

Site-directed mutagenesis. The 1.1-kilobase (kb) *Bgl*II-*Sph*I fragment from pUC160 (7) was inserted into *Bam*HI-*Sph*I-digested M13mp19 DNA, and site-directed mutagenesis was performed as described previously (20, 65). In brief, the phage were passed twice through *E. coli* RZ1032 (20). Single-stranded uracil-rich DNA was prepared and hybridized in vitro either to a fivefold molar excess of both the oligonucleotide containing the desired base change(s) and the M13mp19 universal sequencing primer 1211 (New England BioLabs, Inc., Beverly, Mass.) or to two different oligonucleotides, each of which contained base changes in the *abl* sequence (65). Second-strand synthesis was allowed to proceed overnight at 16°C, and a portion of the reaction was removed and used to transform *E. coli* JM101. Phage containing the desired mutation were initially identified by plaque hybridization with the oligonucleotide containing the desired base changes as a probe (65). Phage identified in the preliminary screen were analyzed by DNA sequencing by the dideoxy-chain termination method (38) to confirm the presence of the desired mutation. The amino acid substitutions evaluated are diagrammed in Fig. 1.

Viruses. All of the Abelson viruses used in this study were derived from pUC160, an infectious clone of Ab-MLV containing the Ab-MLV P160 genome from pP160 (21)

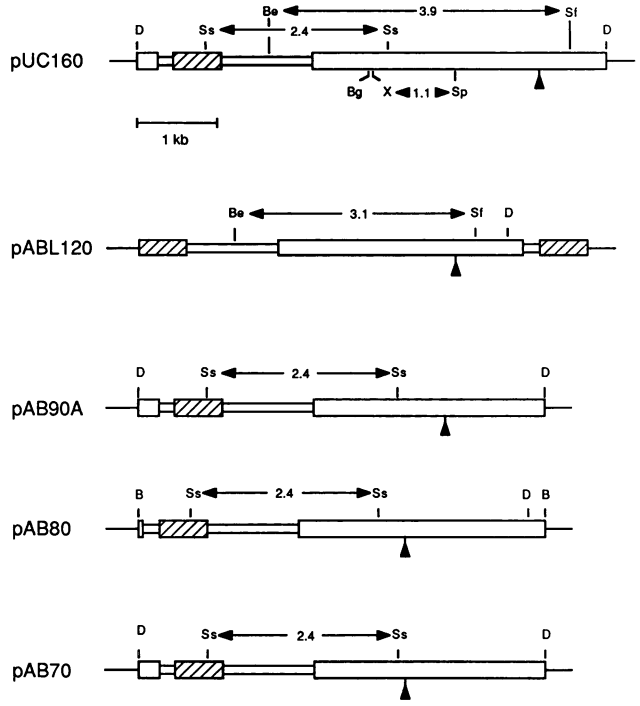

FIG. 2. Ab-MLV plasmids. The structures of the plasmid forms of the Ab-MLV strains used in the experiments are depicted. The thin lines denote pBR322 sequences for pAB90A, pAB80, and pAB70 (13), pUC9 sequences for pUC160 (7), and mink cellular DNA for pABL120 (49). The hatched boxes denote the retrovirus long terminal repeats; the wide boxes denote v-*abl* sequences; the thin boxes denote Moloney murine leukemia virus sequences; vertical arrows mark the position of translation termination codons. Restriction endonuclease site abbreviations: B, *Bam*HI; Be, *Bst*EII; Bg, *Bgl*II; D, *Hin*dIII; Sf, *Sfi*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xmn*I. All distances are in kilobase pairs.

inserted into the *Hin*dIII site of pUC9 (7) (Fig. 2). The sequence of the pP160 isolate of Ab-MLV P160 differs from the published Ab-MLV sequence (29) in that it contains a thymine-to-guanine transversion at position 2184 in the genome, resulting in the substitution of valine for phenylalanine at position 522 in the Abelson protein (unpublished observations). To ensure that all viruses used contained identical long terminal repeats and leader regions, the pUC160 backbone was used to construct all other viruses. The Ab-MLV P120 strain used here was constructed by replacing the 3.9-kb *Bst*EII-*Sfi*I fragment of pUC160 with the corresponding 3.1-kb fragment from pABL120 (49) (Fig. 2). The Ab-MLV P90A, P80, and P70 strains (13) were constructed by replacing the 2.4-kb *Sst*I fragment (bases −35 to 2391) of infectious DNA clones of these viruses with the corresponding fragment from pUC160 (Fig. 2). Because of the construction strategy, the P120 wild-type strain encoded an Abelson protein with a phenylalanine at position 522, whereas all other wild-type and substituted strains encoded proteins with a valine at this position. The valine substitution did not alter the properties of any of the wild-type viruses (our unpublished data); although this substitution affects a region that is highly conserved among protein tyrosine kinases, the proteins encoded by *neu*, *fes*, and *erbB* all have a valine residue at the corresponding position (11).

Viruses containing site-directed mutations were reconstructed in pUC160 by replacing the wild-type 1.1-kb *Xmn*I-*Sph*I fragment with fragments containing the desired mutations (Fig. 2). Other Ab-MLV strains were reconstructed by replacing the 2.4-kb *Sst*I fragment from the plasmid clones of the P120, P90A, P80, and P70 strains of Ab-MLV with the corresponding fragment containing the mutations derived from mutant forms of pUC160. The sequences of all of the plasmids were confirmed by DNA sequencing from bases 2080 to 2500.

The pAB80 plasmid was linearized with *Bam*HI, and all of the other viral plasmids were linearized with *Hin*dIII, and introduced into NIH 3T3 cells by transfection together with Moloney murine leukemia virus DNA from p8.2 (45) or pNCA (23) by calcium phosphate-mediated precipitation (2, 58). Virus was harvested from transformed cells maintained at 34.5°C 6 to 10 days after transfection.

Immunoprecipitation, immunoblotting, and autophosphorylation assays. For in vitro autophosphorylation, cellular extracts were prepared and processed as described elsewhere (59). Abelson proteins were immunoprecipitated by using either the H548 mouse monoclonal antibody (3; gift of B. Chesebro, Rocky Mountain Laboratory, Hamilton, Mont.) that reacts with the $p12^{gag}$ portion of the Abelson protein or the 24-21 mouse monoclonal antibody that reacts with the carboxy-terminal region of the Abelson protein (40). The immunoprecipitated samples were divided in half, and one portion was reacted with [α-^{32}P]ATP by using the standard in vitro phosphorylation protocol (59). In some experiments, acid-denatured enolase was added before the addition of labeled ATP (5). The other half of the sample was processed in an identical fashion, except that isotope was not added to the preparation. All reactions were incubated at 0°C for 10 min. The samples were analyzed by electrophoresis through a 10% sodium dodecyl sulfate–polyacrylamide gel. The gel containing the labeled samples was dried and exposed to Kodak XAR film with an intensifying screen for 10 min to 18 h. Proteins in the other gel were transferred to nitrocellulose filters (52). After transfer, the filters were treated with 0.25% gelatin–0.05% Nonidet P-40–150 mM NaCl–50 mM Tris hydrochloride (pH 7.4)–5 mM EDTA (22), reacted with appropriate monoclonal antibodies overnight, and developed with goat anti-rabbit immunoglobulin G conjugated to peroxidase (Organon Teknika-Cappel, Malvern, Pa.).

For analysis of phosphotyrosine, cells were labeled with [^{35}S]methionine as previously described (61). Cells were lysed in 1% Triton X-100–10 mM Tris (pH 7.6)–5 mM EDTA–50 mM NaCl–50 mM sodium fluoride–100 μM sodium orthovanadate–0.1% bovine serum albumin–0.02% sodium azide–1 mM phenylmethylsulfonyl fluoride. The extracts were adjusted to contain equal amounts of trichloroacetic acid-precipitable radioactivity and divided into two portions. One half of the sample was precipitated with the anti-$p12^{gag}$ monoclonal antibody H548 (3), and the other half was precipitated with the Sepharose-linked mouse monoclonal antibody 1G2 (gift of R. Frackelton, Brown University, Providence, R.I.), an antibody that reacts with phosphotyrosine (14). The samples precipitated with H548 were processed as previously described (61). The samples precipitated with 1G2 were washed and eluted with 1 mM phenylphosphate before fractionation through 10% sodium dodecyl sulfate–polyacrylamide gels.

Phosphoamino acid analysis. For analysis of total phosphoamino acids, cells were labeled with carrier-free $^{32}P_i$ at a concentration of 1 mCi/ml for 18 h in phosphate-free DMEM supplemented to contain 10% dialyzed new born calf serum. The cells were lysed in 1.0% Triton X-100–10 mM

$Na_2HPO_4 \cdot NaH_2PO_4$ (pH 7.5)–0.5% sodium deoxycholate–0.1% sodium dodecyl sulfate–100 mM NaCl–0.02% sodium azide (61). Phosphoproteins were prepared as described by Hunter and Sefton (16). Briefly, the extracts were centrifuged at 15,000 × *g* at 4°C for 15 min, and phosphoproteins were extracted by adding an equal volume of phenol saturated with NTE (100 mM NaCl, 50 mM Tris [pH 7.5], 5 mM EDTA). The first aqueous phase was extracted with an equal volume of phenol, and then both phenol phases were extracted three times with 2 volumes of NTE. After the final extraction, the phenol phase was diluted in H_2O and the proteins were precipitated by the addition of trichloracetic acid to a final concentration of 15%. The proteins were washed twice with chloroform-methanol (2:1), dissolved in 6 M HCl by heating at 100°C for 1 min, and hydrolyzed by heating at 110°C for 2 h. Acid hydrolysates were dissolved in a marker mixture containing 1 mg each of phosphoserine, phosphothreonine, and phosphotyrosine per ml, and the samples were normalized for radioactivity. The samples were analyzed on cellulose thin-layer chromatography plates (Analtech, Inc., Newark, Del.) by electrophoresis at pH 1.9 for 2 to 3 h at 1,000 V in glacial acetic acid–88% formic acid–H_2O (78:25:897). Ascending chromatography was performed in isobutyric acid–0.5 M NH_4OH (5:3). The markers were detected by staining with ninhydrin.

RESULTS

Effect of the point mutations on Ab-MLV transformation. Our previous studies suggested that Abelson proteins containing either the His-590 or the Gly-536 substitution were compromised in kinase activity at high temperatures (7). However, the His-590 substitution failed to induce temperature-sensitive transformation when expressed in a wild-type Abelson protein. Also, the effect of the Gly-536 mutation on viral transformation was not analyzed at the time of our earlier report (7). To more fully understand the impact of these two substitutions on Ab-MLV transformation, a panel of Ab-MLV strains containing each of the missense mutations was constructed. In addition, two strains were constructed that contained both mutations. The panel included both the P120 and P160 wild-type strains as well as the P70, P80, and P90A strains. These latter three viruses are derived from the P120 strain and encode Abelson proteins lacking 357, 293, and 195 carboxy-terminal amino acids, respectively (13, 25; Fig. 1).

The loss of transformed phenotype at the nonpermissive temperature is a hallmark of *ts* transformation mutants. To determine which of the mutations induced temperature-dependent transformation, the growth pattern of individual transformed foci of NIH 3T3 cells was compared at 34.5 and 39.5°C. The cells were considered to display temperature-sensitive transformation if they became flat and adhered to the surface of the tissue culture dish within the first 24 to 48 h of growth at the high temperature. Consistent with a large body of work from several laboratories (7, 17, 30), none of the cells transformed by the unmutated P160, P120, P90A, or P80 strain displayed temperature-dependent transformation.

As we had shown previously (7), the P70/H590 strain displayed temperature sensitivity in NIH 3T3 cells (Table 1). Surprisingly, one of nine clones transformed with the unmodified P70 strain displayed temperature-dependent transformation; thus, this strain may be slightly compromised in its ability to maintain transformation at the restrictive temperature. In contrast to the results obtained with the P70/H590 strain, mass populations of NIH 3T3 cells expressing P80/H590 did not revert to normal morphology upon growth at a high temperature (data not shown). This result and the observation that neither of the two foci examined reverted after temperature shift suggest that the addition of as few as 64 v-*abl*-encoded amino acids at the carboxyl terminus of the P80 protein can mask the effect of the His-590 mutation. None of the clones transformed with the P90, P120, or P160 strain carrying this mutation displayed conditional transformation.

TABLE 1. Temperature-sensitive transformation by Ab-MLV strains[a]

Virus	No. displaying *ts* transformation/total	
	NIH 3T3 cells	Lymphoid cells
P160	0/4	0/2
P160/H590	0/3	0/9
P120	0/12	0/2
P120/H590	0/3	0/6
P120/G536	4/10	8/8
P120/G+H	26/26	10/10
P90A	0/11	NT
P90A/H590	0/12	NT
P90/G536	0/7	NT
P80	0/7	NT
P80/H590	0/2	NT
P70	1/9	0/2
P70/H590	13/15	30/30
P70/G536	14/15	2/2

[a] Individual foci of transformed NIH 3T3 cells and individual colonies of transformed lymphoid cells were expanded and maintained at 34.5°C until stable cell lines were obtained. To assess temperature-dependent transformation, parallel cultures were plated at 34.5 and 39.5°C and observed for 48 h. NIH 3T3 cells were considered temperature sensitive for transformation if the cells assumed a flat morphology at the high temperature. Lymphoid cells were considered temperature sensitive for transformation if they failed to survive at the high temperature. NT, Not tested.

The Gly-536 substitution induced temperature-dependent transformation in the context of the P70 protein but did not appear to affect transformation by the P90A strain. However, 4 of 10 foci transformed by the P120/G536 strain were *ts* (Table 1). The viral protein expressed in these clones was indistinguishable in size from that expressed by clones that remained transformed at the high temperature (data not shown), suggesting that gross changes in the protein were not involved in this phenomenon. The Gly-536 substitution was not tested in the context of the P80 or P160 proteins.

In addition to viruses containing the single missense mutation, two strains, designated P120/G+H and P70/G+H, were constructed that contained both mutations. In contrast to the single substitutions, all transformed clones derived from the P120/G+H strain displayed temperature-dependent transformation. Despite repeated attempts to recover transformed foci, the P70/G+H strain failed to transform NIH 3T3 cells at the permissive temperature. This last result again highlights the slightly defective nature of the unmodified P70 protein.

Ab-MLV-transformed pre-B cells isolated by using the *ts* viruses survive for only 24 to 48 h at the nonpermissive temperature (7a). To determine whether the viruses that induced temperature-dependent transformation in NIH 3T3 cells behaved in a similar fashion in pre-B cells, a panel of clones were derived at 34.5°C and monitored for growth at low and high temperature. In general, viruses that displayed *ts* transformation in the NIH 3T3 cells were also *ts* in lymphoid cells (Table 1). However, whereas fewer than half of the NIH 3T3 clones transformed by the P120/G536 strain

TABLE 2. Titration of Ab-MLV strains on NIH 3T3 cells[a]

Virus and stock	Titer (FFU/ml)		Titer at 39.5°C/ titer at 34.5°C ratio
	34.5°C	39.5°C	
P160			
1	2.5×10^5	4.0×10^5	1.6
2	2.6×10^5	5.1×10^5	2.0
P160/H590			
1	3.4×10^5	5.6×10^5	1.7
2	1.7×10^5	3.2×10^5	1.9
P120			
1	4.4×10^5	6.8×10^5	1.6
2	4.1×10^5	3.3×10^5	0.8
P120/H590			
1	2.0×10^6	2.0×10^6	1.0
2	1.4×10^6	1.5×10^6	1.1
P120/G536			
1	2.3×10^4	7.0×10^3	0.3
2	4.5×10^4	4.1×10^3	0.2
P120/G+H			
1	1.4×10^4	$<1.0 \times 10^1$	<0.0007
2	1.9×10^5	7.0×10^1	0.0004
P70			
1	1.2×10^5	5.5×10^4	0.5
2	3.0×10^5	1.1×10^5	0.4
P70/H590			
1	4.0×10^5	1.4×10^4	0.03
2	7.0×10^5	1.0×10^4	0.014
P70/G536			
1	2.1×10^4	8.6×10^2	0.041
2	3.4×10^4	1.4×10^3	0.041

[a] The titers of the Ab-MLV strains were determined by using the NIH 3T3 transformation assay (39).

TABLE 3. Lymphoid transformation by Ab-MLV strains[a]

Virus and expt	No. of foci/10^6 cells		Foci at 39.5°C/foci at 34.5°C ratio
	34.5°C	39.5°C	
P160			
1	220	180	0.8
2	55	14	0.3
P120			
1	62	101	1.6
2	31	107	3.5
P120/G536			
1	60	<0.5	<0.009
1	51	<0.5	<0.010
P120/G+H			
1	59	<0.5	<0.009
2	54	<0.5	<0.010
P70			
1	32	17	0.5
2	5	2	0.4
P70/H590			
1	108	<0.5	<0.005
2	100	<0.5	<0.005
P70/G536			
1	35	<0.5	<0.014
2	40	<0.5	<0.013

[a] Lymphoid cell transformation was monitored using the agar transformation assay (34). Approximately equal numbers of fibroblast focus-forming units were used for all infections. The values shown represent the number of transformed pre-B cell colonies obtained per 10^6 nucleated bone marrow cells.

were temperature sensitive, all eight pre-B cell clones displayed this property. These data suggest that the requirements for lymphoid and NIH 3T3 cell transformation are distinct. The lymphoid cells may require higher levels of protein tyrosine kinase activity. Alternatively, interaction with different substrates may be important in the two cell types.

NIH 3T3 transformation assays (39) were carried out at the permissive and nonpermissive temperatures to measure the fibroblast focus-forming units in some of the virus stocks. Either of the substitutions reduced the titer of P70 strains at the nonpermissive temperature, and the combination of the two mutations drastically affected the titer of the P120 strain at the high temperature (Table 2). Consistent with the subtle effect on the growth of NIH 3T3 cells transformed by the unmodified P70 strain, this strain showed a slight reduction in titer at the nonpermissive temperature. The P160 and P120 strains carrying the His-590 substitution had identical titers on NIH 3T3 cells at both temperatures (Table 2).

The agar transformation assay (34) was used to measure the frequency of lymphoid colonies induced by the viruses at both temperatures. The P70/H590, P70/G536, P120/G536, and P120/G+H strains all failed to transform lymphoid cells at the nonpermissive temperature (Table 3). Preliminary experiments suggest that the P160/G536 strain is also unable to transform pre-B cells at the restrictive temperature (data not shown). The inability of the P120 and P160 strains carrying the Gly-536 substitution to transform pre-B cells at the nonpermissive temperature is consistent with the inability of these viruses to maintain pre-B cell transformation at 39.5°C.

Missense mutations reduce kinase activity in vitro. The kinase activity of Abelson protein can be assessed in several ways in vitro. The autophosphorylation immune complex assay reflects both the activity of the protein and its ability to serve as a substrate (59). Second, this assay can be modified to monitor phosphorylation of substrates such as enolase, a reaction that reflects enzymatic activity (5). Finally, because Abelson proteins of several different sizes are available, phosphorylation patterns in extracts containing mixtures of two different proteins can be used to assess the ability of one of them to function as a substrate (63). We examined the various Abelson proteins in these three assays to determine how parameters of kinase activity were affected by the substitutions.

In the first series of experiments, parallel cultures of lymphoid cells infected with the panel of P70 and P120 strains were grown for 1 day at 34.5 and 39.5°C, and the cells were processed for the in vitro autophosphorylation reaction (59). Immunoblotting of a sample of each immunoprecipitation revealed that some clones consistently expressed less Abelson protein than others. However, individual clones expressed about the same amount of Abelson protein irrespective of the temperature at which they were grown (Fig. 3A). Despite similar levels of Abelson protein, the kinase

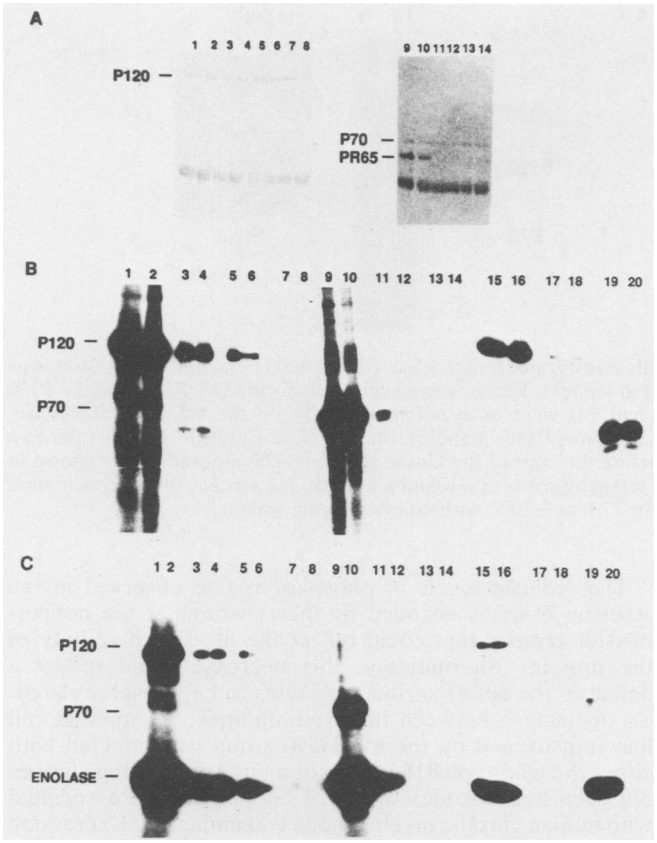

FIG. 3. In vitro phosphorylation assays of Abelson proteins. Duplicate cultures of lymphoid cells transformed by Ab-MLV P120 (lanes 1 and 2, 15 and 16), P120/H590 (lanes 3 and 4, 17 and 18), P120/G536 (lanes 5 and 6), P120/G+H (lanes 7 and 8), P70 (lanes 9 and 10, 19 and 20), P70/H590 (lanes 11 and 12), and P70/G536 (lanes 13 and 14) were grown for 1 day at 34.5°C (odd-numbered lanes) or 39.5°C (even-numbered lanes). The cells were lysed and prepared for the in vitro autophosphorylation reaction as described in the text. (A) Western blot analysis of a portion of the reaction. Nitrocellulose filters were developed with goat anti-rabbit immunoglobulin G conjugated to peroxidase after incubation with the anti-v-*abl* monoclonal antibody 24-21 (lanes 1 through 8) or the anti-p12 monoclonal antibody H548 (lanes 9 through 14). (B) Analysis of the in vitro autophosphorylation reaction performed on a portion of the sample analyzed in panel A as described in the text. The lane designation is identical to that in panel A. (C) In vitro autophosphorylation reaction in the presence of acid-denatured enolase performed as described in the text. The lane designations are the same as described for panel A. In panels B and C, lanes 1 through 14 were exposed to Kodak XAR-4 film for 4 h at −70°C with a Kronex intensifying screen. Lanes 15 through 20 are identical to lanes 1 through 4, 9, and 10, except that they were exposed for 10 min at room temperature.

activity of all of the substituted Abelson proteins was reduced compared with that of the wild type even when the cells were grown at the permissive temperature (Fig. 3B).

The magnitude of the difference in kinase activity observed between proteins encoded by the wild-type and *ts* strains varied with the extraction conditions. However, the relationship of the proteins to each other was similar to that in Fig. 3 when buffers lacking ionic detergents were used (data not shown). In the experiment shown, estimates of kinase activity based on scanning densitometry revealed that the P120/H590 protein (Fig. 3, lanes 3 and 4) had about fivefold less activity than the wild-type protein (lanes 1 and 2). The activity of the P120/G536 protein (lanes 5 and 6) and doubly substituted protein (lanes 7 and 8) was reduced about 15- and 200-fold, respectively. For the P70 proteins, the His-590 substitution (lanes 11 and 12) lowered activity about 30-fold, whereas the Gly-536 substitution (lanes 13 and 14) reduced activity over 100-fold. For the *ts* viruses, the kinase activity of proteins extracted from cells grown at high temperature was often reduced severalfold compared with that seen in extracts prepared from cells maintained at the low temperature. Consistent with our previous results (7), this reduction was particularly evident for P70/H590 (lanes 11 and 12). When the kinase assay was carried out in the presence of acid-denatured enolase (Fig. 3C), results similar to those obtained in the earlier protocol were found. Thus, the decreased labeling of Abelson proteins reflects their kinase activity.

To determine whether the lowered phosphorylation observed with the various substituted forms of Abelson protein reflected only kinase activity and not the ability of the molecules to function as substrates, we examined phosphorylation of mixtures of wild-type and substituted proteins (63). When extracts containing mixtures of wild-type proteins were reacted in the standard kinase assay, both proteins were well labeled (Fig. 4, lanes 3 and 5). However, when a wild-type P160 protein was mixed with either P70/G536 or P120/G+H, the P160 protein was well labeled but the intensity of the signal obtained with the substituted proteins was barely visible (lanes 6 and 7, overexposed in lanes 10 and 11, and data not shown). Indeed, this signal did not differ from that obtained when the substituted proteins were analyzed alone (lanes 8 and 9, overexposed in lanes 12 and 13). Similar results were obtained when the extracts from the *ts*-transformed cells were heat denatured before the reaction (data not shown). Taken together with the previous results, these data indicate that the substituted proteins are compromised in both their enzymatic activity and their ability to be phosphorylated on tyrosine.

Mutant forms of the P70 and P120 proteins differ in phosphotyrosine in vivo. For Abelson proteins, the sites of tyrosine phosphorylation observed in the in vitro kinase reaction are distinct from those observed in vivo (19, 44). To determine whether the lowered in vitro kinase activities reflected the relative levels of tyrosine phosphorylation in vivo, the total cellular phosphotyrosine in cells transformed by the P70 and P120 series of viruses was assessed (data not shown). Consistent with earlier reports (44), transformation was accompanied by a four- to sixfold increase in phosphotyrosine, and this species accounted for about 0.4 to 0.6% of the total phosphoamino acids recovered from all of the transformed cells. This level decreased less than twofold when cells transformed by wild-type strains were maintained at the nonpermissive temperature. However, reductions of an additional twofold were observed for cells transformed by the P120/G+H strain. Reductions of fourfold were observed for cells transformed by the P70/H590 strain. Thus, for P70/H590, the levels observed at the nonpermissive temperature approached those seen in control cells.

To assess the amount of phosphotyrosine on the Abelson proteins recovered from cells grown at the two temperatures, NIH 3T3 cells transformed by the P70 strains and the P120 wild-type and P120/G+H strains were analyzed. The cells were labeled with [^{35}S]methionine and immunoprecipitated with either anti-Abelson protein or anti-phosphotyrosine antibodies (Fig. 5). As in the earlier analyses, each cell line contained a characteristic amount of Abelson pro-

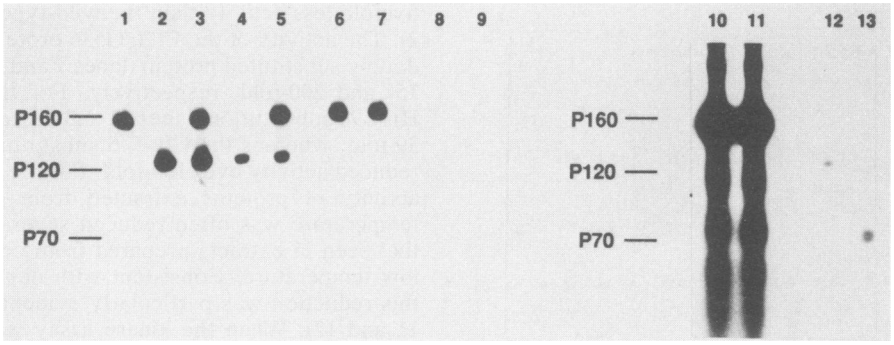

FIG. 4. In vitro phosphorylation in the presence of P160. Lymphoid cells transformed by the P160, P120, P120/G+H, and P70/G536 strains were processed for the in vitro autophosphorylation reaction as described in the text. Extracts from cells transformed by P160 (lane 1), P120 (lanes 2 and 4), P120/G+H (lanes 8 and 12), and P70/G536 (lanes 9 and 13) were analyzed individually by the standard procedure. Alternatively, extracts from cells transformed by either the P160 and P120 strains (lanes 3 and 5), the P160 and P120/G+H strains (lanes 6 and 10), or the P160 and P70/G536 strains (lanes 7 and 11) were mixed before the start of the kinase reaction. The autoradiogram shown in lanes 1 through 9 was exposed to Kodak XAR-4 film for 20 min at room temperature with a Kronex intensifying screen; the autoradiogram shown in lanes 10 through 13 represents exposure of lanes 6 through 9 for 17 h at −70°C with an intensifying screen.

tein and the level of protein did not vary with temperature (Fig. 5A). The amount of phosphotyrosine-containing Abelson protein in both forms of substituted P70 and in P120/G+H was decreased about 1.5- to 3-fold when the cells were maintained at the nonpermissive temperature (Fig. 5B, lanes 4, 6, and 10). The level of phosphotyrosine recovered in the P70/G536 protein was reduced compared with that observed in the unmodified P70 protein at the permissive temperature and further reduced at the nonpermissive temperature (Fig. 5B, lanes 1, 3, and 4). Consistent with the total phosphotyrosine analysis, the amount of phosphotyrosine in these substituted proteins was similar to that in the unmodified Abelson proteins at the permissive temperature (Fig. 5B, lanes 1, 5, 7, and 9).

The reduced levels of phosphotyrosine observed in the Abelson proteins encoded by the *ts* strains at the nonpermissive temperature could reflect the decreased activity of the protein. Alternatively, this decrease could reflect a defect in the ability of the molecules to be phosphorylated. To distinguish between these possibilities, a lymphoid cell line transformed by the P70/H590 strain was infected with either the wild-type P160 strain or a virus expressing another *abl* gene that encodes the P120 *bcr/abl* protein associated with human chronic myelogenous leukemia (18). Expression of either P160 or P210 allowed survival of the cells at the nonpermissive temperature (Engelman and Rosenberg, submitted). Thus, it was possible to analyze the phosphotyrosine levels in both *abl* proteins at the nonpermissive and

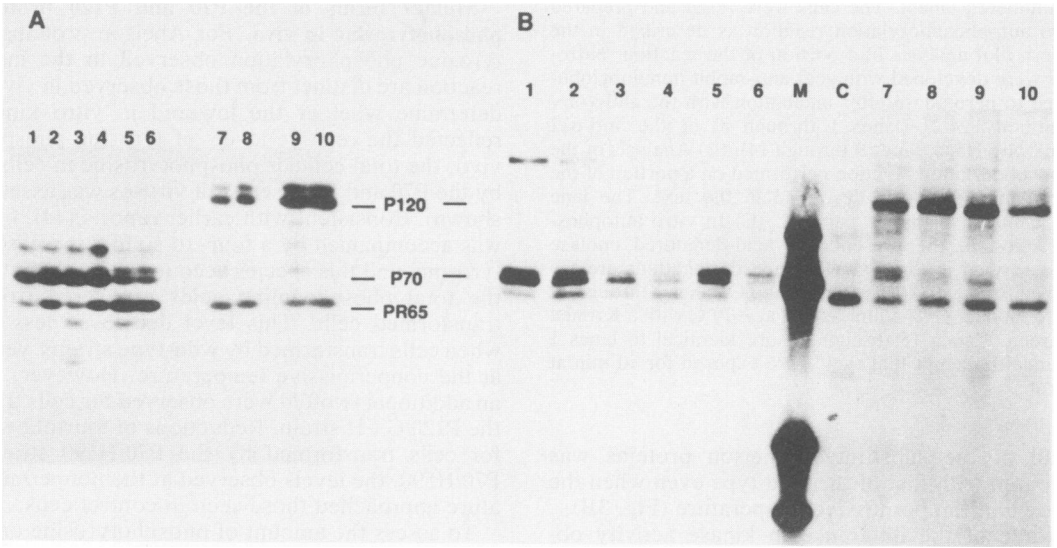

FIG. 5. Phosphorylation of Abelson proteins in vivo. (A) Duplicate cultures of NIH 3T3 cells transformed by Ab-MLV P70 (lanes 1 and 2), P70/G536 (lanes 3 and 4), P70/H590 (lanes 5 and 6), P120 (lanes 7 and 8), or P120/G+H (lanes 9 and 10) were grown overnight at 34.5°C (odd-numbered lanes) or 39.5°C (even-numbered lanes). Extracts from [^{35}S]methionine-labeled cells were immunoprecipitated with the anti-p12 monoclonal antibody (H548) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the text. The gel was exposed to XAR-4 film for 4 days at −70°C. (B) Portions of the samples analyzed in panel A were immunoprecipitated with the anti-phosphotyrosine monoclonal antibody 1G2 (14). The gel was exposed to XAR-4 film for 2 weeks at −70°C. Other lanes: M, molecular weight standards; C, proteins immunoprecipitated from NIH 3T3 cells.

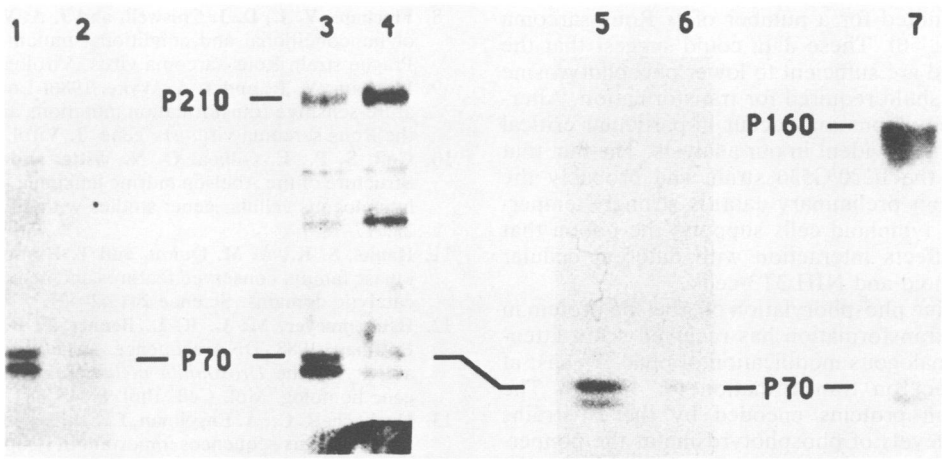

FIG. 6. Phosphorylation of *abl* proteins in cells expressing P210 and P70/H590. Parallel cultures of the 7C411, a pre-B-cell clone transformed by the P70/H590 strain, were grown at 34.5°C (lanes 1, 3, and 5) or 39.5°C (lanes 2, 4, 6, and 7). The cells shown in lanes 3 and 4 had been infected with a virus expressing P210 *bcr/abl* (24), and the cells shown in lane 7 were infected with the P160 wild-type virus. The cells were labeled and processed as described in the legend to Fig. 5 for the 1G2 monoclonal antibody.

permissive temperatures. This analysis revealed that the phosphotyrosine level in the P70/H590 protein was not affected by the presence of either P160 or P210 (Fig. 6, lanes 2, 4, 6, and 7). The inability of these proteins to phosphorylate the P70/H590 protein in vivo suggests that tyrosine phosphorylation of Abelson protein may be important in regulating its function.

DISCUSSION

Transfer of mutations by site-directed mutagenesis. Our ability to create *ts* Ab-MLV strains by using sequence information from *ts* RSV strains reinforces our earlier studies demonstrating that transfer of a mutation between related gene family members is an effective way to construct a mutant virus with a predictable phenotype (7). However, success with this approach appears to be strongly dependent on the relatedness of the proteins affected. This point is particularly evident in the case of the His-590 substitution, a change that resulted in a *ts* mutant only when expressed in the context of the P70 protein. Two features of Ab-MLV P70 may contribute to its utility in these studies. First, the P70 protein has an overall similarity to pp60$^{v\text{-}src}$; these two molecules diverge from the protein tyrosine kinase consensus sequence at the same point (11, 29) and each continues for a short stretch of unique amino acids (7, 15). Second, the P70 protein terminates very close to the point defining the minimal transforming sequence of Abelson proteins (1, 27, 28, 30, 55), suggesting that the P70 strain may already be partially impaired in its ability to transform cells. Consistent with this idea, the results of both the NIH 3T3 and lymphoid transformation assays suggest that the unaltered P70 strain is slightly compromised in transformation at the nonpermissive temperature.

The first two mutations we studied gave rise to *ts* Ab-MLVs, suggesting that our method may have general applicability. Nonetheless, it is difficult to predict how often mutations will fit the pattern established by these precedents. The technique requires identification of amino acids that are conserved between the two proteins under study. However, this is not sufficient to ensure success. For example, viruses encoding an Abelson protein in which alanine has been substituted for Pro-605 (57) are not *ts* for transformation (Y. Y. Chen, A. Engelman, and N. Rosenberg, unpublished data). This substitution involves replacement of an amino acid that is found in virtually all protein tyrosine kinases (11), suggesting that the residue plays an important role in protein structure and function. Despite this feature, a change at this position has drastic effects on pp60$^{v\text{-}src}$ and appears neutral in Abelson proteins.

Carboxyl terminus of Abelson protein affects the *ts* phenotype. The carboxyl-terminal domain of the Abelson protein has a strong effect on the ability of the Ab-MLV strains to induce temperature-dependent transformation. The ability of this region to mask the effects of substitutions in the kinase domain is consistent with the idea that the carboxyl terminus stabilizes other parts of the protein and at least indirectly affects the kinase domain. Despite this, the carboxyl terminus is not absolutely required for any of the biological effects of the virus (28). Indeed, among c-*abl* proteins, this region is least conserved (12, 29, 36, 46). The carboxyl-terminal sequences do affect the lethality and the efficiency of lymphoid transformation of Ab-MLV (25, 36, 56, 64), perhaps by affecting kinase activity (36, 56). However, our results with the P70 protein suggest that removal of all carboxyl-terminal amino acids does not affect in vitro kinase activity. The role of the carboxyl-terminal region in Abelson protein function deserves further study.

Protein tyrosine kinase activity and conditional transformation. The protein tyrosine kinase activity of Abelson protein plays a central role in transformation (27, 30, 31, 60). Thus, temperature-dependent alterations in this property might be expected in the *ts* strains. The kinase activity of the proteins encoded by the *ts* strains is low when assayed in vitro. However, this change is temperature independent, suggesting that it reflects the inherent instability of the substituted proteins. The decreased activity is evident even in the absence of ionic detergents. This result distinguishes the effects observed here from those noted both for c-*abl* proteins (19) and for some pp60$^{v\text{-}src}$ proteins encoded by *ts* Rous sarcoma virus strains (42).

The in vivo analyses revealed subtle changes in total cellular phosphotyrosine at the nonpermissive temperature. However, the levels only approached those observed in normal cells for one of the two mutations tested. Similar

results have been noted for a number of *ts* Rous sarcoma virus strains (4, 43, 50). These data could suggest that the reductions observed are sufficient to lower phosphotyrosine levels below a threshold required for transformation. Alternatively, drastic reductions may occur in particular critical substrates that are not evident in our analysis. The fact that transformation by the P120/G536 strain and probably the P160/G536 strain (our preliminary data) is strongly temperature dependent in lymphoid cells supports the notion that this substitution affects interaction with different cellular molecules in lymphoid and NIH 3T3 cells.

The role of tyrosine phosphorylation of Abelson protein in Ab-MLV-induced transformation has received scant attention. Indeed, the analogous modification of pp60$^{v\text{-}src}$ has, at best, a subtle effect on transformation (6, 47, 48). The substituted Abelson proteins encoded by the *ts* strains display decreased levels of phosphotyrosine at the nonpermissive temperature. The presence of active *abl*-encoded protein tyrosine kinase fails to correct this defect, suggesting either that impaired kinase activity alone is not responsible for the deficiency or that tyrosine phosphorylation in vivo is an intramolecular reaction. The reduced levels could reflect altered intracellular localization of the substituted proteins at the nonpermissive temperature. Alternatively, conformational changes that may be important for function could be involved. Phosphorylation of the polyomavirus middle-T antigen on tyrosine has recently been shown to be important for appropriate interaction of the middle-T pp60$^{c\text{-}src}$ complex with phosphatidylinositol 3-kinase (51). Our data suggest that the role of phosphotyrosine-modified Abelson protein in transformation deserves attention.

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