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Genetics

Isolation of temperature-sensitive Abelson virus mutants by site-directed mutagenesis

(v-*abl*/v-*src*/protein-tyrosine kinase/transformation)

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ABSTRACT **Mutants of Abelson virus encoding temperature-sensitive protein-tyrosine kinase (EC 2.7.1.112) were created by site-directed mutagenesis using sequence information from temperature-sensitive mutants of the related v-*src* oncogene. Expression of these two independent mutations in *Escherichia coli* resulted in reduced phosphorylation of the mutant proteins at high temperature. Viruses containing one of the mutations induced conditional transformation of both NIH 3T3 and lymphoid cells when expressed in the context of a truncated transforming protein. These results underscore the functional homology between protein-tyrosine kinases and suggest that transfer of mutations within a related gene family may provide a rapid method to create mutants.**

Abelson murine leukemia virus (Ab-MuLV) is a rapidly transforming, replication-defective retrovirus that arose by recombination of Moloney murine leukemia virus (Mo-MuLV) and the protooncogene c-*abl* (1). The virus encodes a single protein containing amino-terminal Mo-MuLV *gag* gene determinants fused to v-*abl*-encoded determinants (2, 3). This molecule is a protein-tyrosine kinase (EC 2.7.1.112), and a portion of the protein shares extensive homology with other protein-tyrosine kinases, including a number of oncogene products and growth factor receptors (4–6). Expression of an enzymatically active Ab-MuLV protein is required for transformation of both lymphoid and fibroblast cells *in vitro* and for tumor induction *in vivo* (7, 8).

Studies addressing the role of protein-tyrosine kinases in transformation have been facilitated by the availability of conditional mutants. In addition, because transformation often appears to block differentiation, some of these mutants have served as useful tools to analyze the events involved in maturation of some cell types (9, 10). Among the viruses that encode protein-tyrosine kinases, temperature-sensitive (ts) mutants of Rous sarcoma virus (RSV) (11–14), of Fujinami sarcoma virus (15), of avian erythroblastosis virus (9) and recently of Ab-MuLV (16, 17) have been reported. With the exception of one series of Ab-MuLV mutants, these mutants have been selected using biological approaches that are generally labor intensive. In addition, the approaches used cannot be readily applied to other retroviruses that encode related proteins.

The functional similarity and homology between protein-tyrosine kinases suggested that sequence information from one ts mutant could possibly be used to engineer ts lesions into a different, but related, protein. We tested this hypothesis by introducing the point mutations present in two ts RSV mutants into the genome of Ab-MuLV. Expression of the kinase region of v-*abl* containing these mutations in *E. coli* resulted in reduced tyrosine phosphorylation of the protein at high temperature. One of the mutant-containing fragments has been used to construct infectious Ab-MuLV. Expression of the mutation in the truncated Ab-MuLV-P70 strain conferred a ts phenotype as judged by the failure of this virus to transform NIH 3T3 cells at the nonpermissive temperature. In addition, NIH 3T3 cells transformed at the permissive temperature reverted to a normal, flat morphology at the nonpermissive temperature, and the kinase activity of P70 protein prepared from these cells after growth at the nonpermissive temperature was reduced. These data underscore the functional similarity between protein-tyrosine kinases and suggest that transfer of mutations within a related gene family may provide a rapid method to isolate conditional mutants.

MATERIALS AND METHODS

Cells and Viruses. Ab-MuLV strains were grown in NIH 3T3 cells (8). pUC160 is an infectious clone of Ab-MuLV containing the Ab-MuLV-P160 genome from pP160 (18) inserted into the *Hin*dIII site of pUC9 (Fig. 1*A*). Ab-MuLV-P70 was derived from pAb1Ab1 (ref. 20; Fig. 1*A*). Nucleotides 2462 and 2463 are deleted in this virus, causing premature termination of translation 32 bases downstream of the deletion (unpublished data). DNA from these plasmids and Mo-MuLV DNA from p8.2 (21) was introduced into NIH 3T3 cells by transfection (22, 23).

The *tsLA25* RSV mutant, a gift of J. Wyke (Imperial Cancer Research Fund Laboratories, London, U.K.), had previously been shown to carry mutation(s) responsible for ts transformation between nucleotides 8210 and 8570 of the RSV genome (24). A clone of the virus was prepared by inserting unintegrated viral DNA into λL47.1 (25, 26). The temperature sensitivity of the clone was confirmed by virus rescue after cotransfection with a replication-defective clone of the avian leukosis virus NTRE-4 (ref. 26; unpublished data). To identify the mutation in *tsLA25*, the *Sst* I–*Eco*RI fragment (bases 6865 to 9238) was subcloned into a Bluescribe vector (Stratagene Cloning Systems, San Diego, CA), and the sequence between bases 8200 and 8635 was determined on both strands using synthetic oligonucleotide primers and the dideoxynucleotide chain-termination method (27).

Site-Directed Mutagenesis. The 1.1-kb *Bgl* II–*Sph* I fragment from pUC160 was inserted into *Bam*HI/*Sph* I-digested M13mp19 DNA, and site-directed mutagenesis was done as described (28, 29). The phage containing the desired mutation were identified by DNA sequencing using the dideoxy chain-termination method. Ab-MuLV-P160 was reconstructed by replacing the 1.1-kb *Xmn* I–*Sph* I fragment of the wild-type pUC160 plasmid with the corresponding mutant-containing fragment. Ab-MuLV-P70 mutants were constructed by replacing the wild-type 2.4-kb *Sst* I fragment with the

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Abbreviations: ts, temperature sensitive; Ab-MuLV, Abelson murine leukemia virus; RSV, Rous sarcoma virus; Mo-MuLV, Moloney murine leukemia virus.

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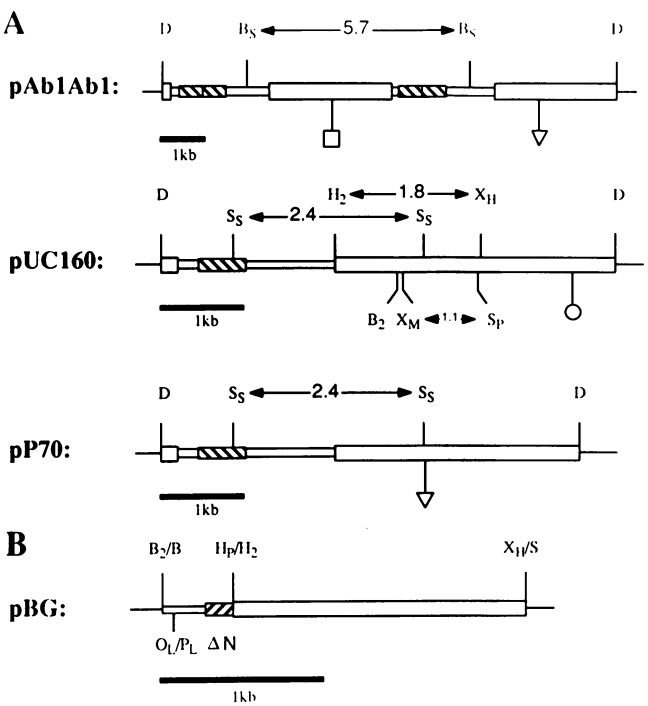

FIG. 1. Retrovirus and bacterial expression plasmids. (*A*) Ab-MuLV-P70 was derived from pAb1Ab1 by removing the 5.7-kilobase (kb) *Bst*EII fragment and replacing the 2.4-kb *Sst* I fragment with the corresponding fragment from pUC160. Thin lines denote plasmid sequences, hatched boxes denote long terminal repeats, wide boxes denote v-*abl* sequences, and thin boxes denote Mo-MuLV sequences. The location of termination codons are designated by ○, □, and ▽. (*B*) Bacterial expression plasmids (pBG) were constructed by inserting the 1.8-kb *Hin*cII–*Xho* I fragment from wild-type and mutant Ab-MuLV-P160 plasmids into pPL-λ DNA (19). The thin line denotes vector sequences, the hatched box denotes *N* sequences, the wide box denotes v-*abl* sequences, and the thin box denotes λ O_L/P_L sequences. Restriction sites are: B, *Bam*HI; B_2, *Bgl* II; B_s, *Bst*EII; D, *Hin*dIII; H_2, *Hin*cII; H_p, *Hpa* I; S, *Sal* I; S_p, *Sph* I; S_S, *Sst* I; X_H, *Xho* I; and X_M, *Xmn* I. All distances are in kb.

corresponding mutant-containing fragment from the Ab-MuLV-P160 mutant constructs. The sequence of these plasmids was confirmed by DNA sequencing from base 2080 to 2500.

Expression of v-*abl* Sequences in *E. coli*. To study expression of the wild-type and mutant proteins in *E. coli*, the 1.8-kb *Hin*cII–*Xho* I fragment from the Ab-MuLV-P160 constructs was inserted into *Hpa* I/*Sal* I-digested pPL-λ DNA (19) (Fig. 1*B*). This plasmid directs synthesis of a fusion protein containing the amino-terminal 58 amino acids of the λ N protein fused to v-*abl*-encoded determinants.

Protein Analysis. v-*abl*-containing proteins expressed in *E. coli* were labeled with [^{35}S]methionine and [^{32}P]O_4^{2-} and processed for immunoprecipitation (30, 31) with the monoclonal anti-v-*abl* antibody 19–110 (32). [^{35}S]Methionine labeling of eukaryotic cells and the *in vitro* tyrosine phosphorylation assay were done as described (33).

RESULTS

Candidates for Site-Directed Mutagenesis. To identify possible targets for mutagenesis, the deduced protein sequence of P160 (34) was aligned with that of protein pp60 encoded by two ts RSV mutants, *tsLA*25 (24) and *tsLA*31 (35), and a second-site revertant of *tsLA*31, 31A.3.4 (35). Analysis of *tsLA*25 revealed a cytosine-to-guanine transversion at nucleotide 8440 in the viral genome, resulting in the substitution of a glycine for an arginine at position 438 in pp60 (Fig. 2). Inspection of the sequence of P160 in this region reveals homology flanking the mutation with a conservative change at the site of the lesion (Lys-536 in P160 compared to Arg-438 in pp60). Based on this information, site-directed mutagenesis was used to change the lysine codon to a glycine codon to generate Ab-MuLV/G536.

In the second instance, analysis of both a ts RSV and a second-site revertant of this virus was necessary to identify a site for mutagenesis. Published sequence information (35) revealed that *tsLA*31 contains a guanine-to-adenine transition at nucleotide 8561 in the viral genome resulting in the substitution of an aspartic acid for a glycine residue at position 478 in pp60. In the 31A.3.4 revertant, a cytosine-to-thymine transition at nucleotide 8602 causes the substitution of a tyrosine for a histidine at position 492 in pp60. Comparison of P160 and the pp60 proteins encoded by mutant *tsLA*31 and second-site revertant 31.A.3.4 revealed that P160 protein resembled the revertant form of pp60 protein. Following this logic, site-directed mutagenesis was used to change the codon specifying tyrosine 590 to a histidine codon to create the Ab-MuLV/H590 strain.

Phosphorylation of the Altered Abelson Proteins Is Reduced at High Temperature in *E. coli*. Abelson protein expressed in *E. coli* is phosphorylated on tyrosine (37). To determine if the point mutations introduced into Ab-MuLV affected phosphorylation of Abelson protein in a bacterial expression system, a pPL-λ (19) expression system was used (Fig. 1*B*). In this system, N protein is expressed at 42°C in *E. coli* strains carrying a thermolabile C_I repressor (38). Thus, both synthesis and tyrosine phosphorylation of Abelson proteins can be studied at a high temperature. Analysis of [^{35}S]methionine-labeled bacteria carrying the wild-type N/v-*abl* fusion re-

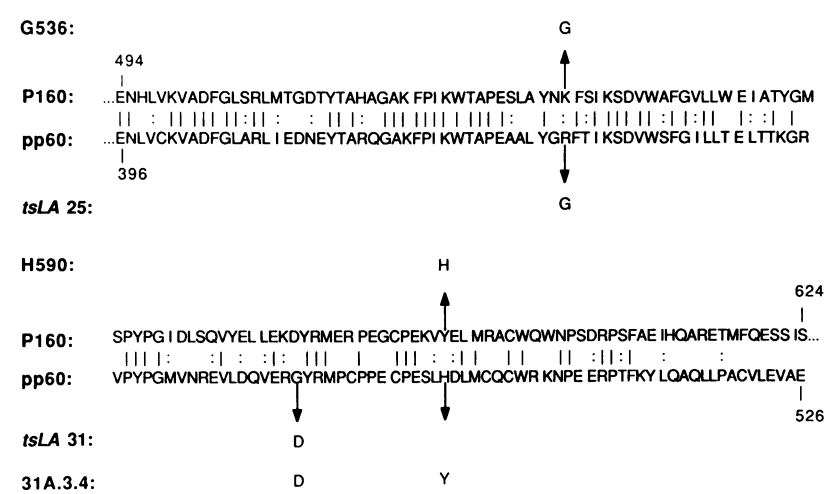

FIG. 2. Mutations in P160 and pp60. The amino acid sequences of wild-type and mutant P160 and pp60 proteins (34, 35) are illustrated. Conserved amino acid substitutions (36) are indicated by a colon (:). Amino acid changes in the mutants *tsLA*25 and *tsLA*31 and the revertant 31A.3.4 are also indicated.

vealed two major peptides containing v-*abl* determinants (Fig. 3*A*, lane 1). The smaller of these is likely to have been generated by proteolysis. Similar peptides containing v-*abl* determinants have been noted in bacterial expression systems by others (37). These peptides were not detected in bacteria containing the control plasmid that lacks Ab-MuLV sequences (lane 4). Both constructs containing the mutant-containing Ab-MuLV also synthesized a v-*abl* protein (lanes 2, 3). However, analysis of cultures labeled with [^{32}P]orthophosphate revealed that the product of the wild-type insert was phosphorylated (Fig. 3*B*, lane 1) at a level at least 10–20 times that of either of the products encoded by the mutant-containing fragments (lanes 2, 3). Phosphoamino acid analysis revealed that levels of phosphotyrosine, the major phosphoamino acid present in Abelson protein synthesized in bacterial expression systems (37), were reduced by at least a factor of 20 in cells carrying the mutant construct (data not shown).

Ab-MuLV-P70/H590 Transformed Cells Revert to a Flat Phenotype at High Temperature. To determine the effect of the mutations on Ab-MuLV transformation, the *Xmn* I–*Sph* I fragment containing one of the mutations, H590, was used to reconstruct an Ab-MuLV-P160 and an Ab-MuLV-P70 strain (see Fig. 1*A*). Each of these viruses and wild-type control viruses was introduced into NIH 3T3 cells by transfection in combination with Mo-MuLV DNA. Five days later, the cells were seeded in agar, and colonies that arose at 34°C were selected and expanded in liquid culture. At 34°C, all of the cell lines displayed characteristics typical of Ab-MuLV-transformed NIH 3T3 cells with a mixture of rounded, refractile cells and elongated adherent cells predominating in the culture. To determine if the cells transformed with Ab-MuLV/H590 exhibited a ts phenotype, cell lines derived from both the Ab-MuLV-P70 and Ab-MuLV-P160 series were shifted to 39.5°C, and the morphology of the cultures was examined 18 hr later. Cells transformed by Ab-MuLV-P70/H590, which exhibited a typical transformed morphology at 34°C (Fig. 4*C*) were flat and grew in a more regular pattern after maintenance at the nonpermissive temperature (Fig. 4*D*). Cells transformed by the wild-type Ab-MuLV-P70 strain maintained a transformed morphology at both temperatures (*A* and *B*) as did cells transformed by both wild-type Ab-MuLV-P160 and Ab-MuLV-P160/H590 (data not shown). Consistent with this, NIH 3T3 cells transfected with Ab-MuLV-P160/H590 gave rise to 500–1000 colonies per μg of DNA at both 34°C and 39.5°C, whereas cells transfected with Ab-MuLV-P70/H590 gave rise to <10 colonies per μg of DNA at 39.5°C and 500–1000 colonies per μg of DNA at 34.0°C. Thus, by both morphological criteria and agar plating efficiency, the H590 mutation in the context of a P70, but not a P160, protein resulted in temperature-dependent transformation.

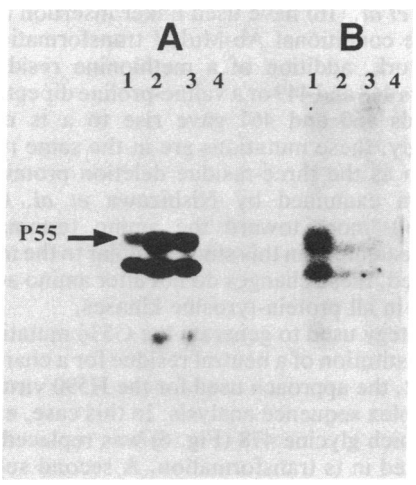

FIG. 3. Expression of N/v-*abl* fusion proteins. pPL-λ plasmids were grown in *E. coli* N4830 (38) at 42°C for 2 hr. (*A*) v-*abl*-encoded determinants in cells containing wild-type (lane 1), G536 (lane 2), and H590 (lane 3) v-*abl* sequences. Lane 4 shows an extract made from cells expressing pPL-λ DNA without insert. Cells were labeled with [^{35}S]methionine (100 μCi/ml; 1 Ci = 37 GBq) for 10 min, and immunoprecipitated extracts (30, 32) were analyzed on a 10% polyacrylamide gel. The gel was exposed to Kodak XAR-4 film for 18 hr. (*B*) Phosphorylation of Abelson protein expressed in *E. coli*. Cells were labeled with [^{32}P]orthophosphate (30 μCi/ml) and analyzed as described in *A*. The gel was exposed to Kodak XAR-4 film for 2 weeks.

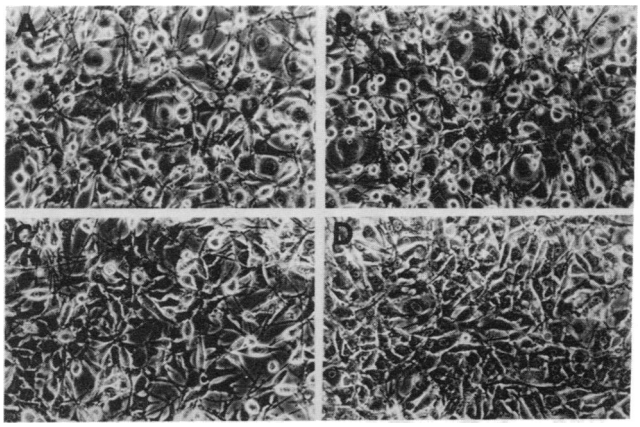

FIG. 4. Morphology of NIH 3T3 cells expressing P70 and P70/H590. Cell lines transformed with Ab-MuLV-P70 (*A* and *B*) and Ab-MuLV-P70/H590 (*C* and *D*) were derived at 34°C. The cells were grown for 18 hr at 39.5°C (*B* and *D*) or 34°C (*A* and *C*) and photographed (×302).

Infection of NIH 3T3 cells with Ab-MuLV-P70/H590 recovered from the transfected cell line passed the ts phenotype to these cells, confirming that the reversion to flat morphology observed in the original transfected cells is a property of Ab-MuLV-P70/H590. In addition, lymphoid cells derived from murine fetal liver cells infected with the P70/H590 virus grew well at 34°C, but these cells died 24–36 hr after the shift to 39.5°C. Similar to the results obtained with NIH 3T3 cells, lymphoid cells transformed by Ab-MuLV-P160/H590 showed no evidence of conditional transformation.

P70/H590 Prepared from Cells at 39.5°C Has Reduced Autophosphorylation Activity *in Vitro*. The H590 mutation conferred a ts phenotype on both NIH 3T3 and lymphoid cells when expressed in the context of the P70, but not the P160, transforming protein. To determine if this change correlated with a reduction in the enzymatic function of the Abelson protein, *in vitro* kinase assays were done using extracts prepared from cells grown at 34°C and 39.5°C. Extracts from NIH 3T3 cells transformed by Ab-MuLV-P70 (Fig. 5*A*, lanes 1–4) displayed equal activity regardless of the temperature at which the cells were grown. Similar results were obtained with lymphoid cells transformed by either Ab-MuLV-P160 (data not shown) or Ab-MuLV-P160/H590 (lanes 11 and 12). In contrast, extracts prepared from either cell type transformed with Ab-MuLV-P70/H590 displayed reduced activities when the cells were maintained at the nonpermissive temperature (compare lanes 5, 7, and 9 with lanes 6, 8, and 10). Pulse–chase analysis using [^{35}S]methionine-labeled cells (Fig. 5*B*) revealed that equivalent amounts of both P160/H590 and P70/H590 are synthesized during the 10-min pulse at 34°C (lanes 1 and 9) and 39.5°C (lanes 5 and 13), and that both are less stable during the 4-hr chase at the nonpermissive temperature (compare lanes 2–4 and 10–12 with lanes 6–8

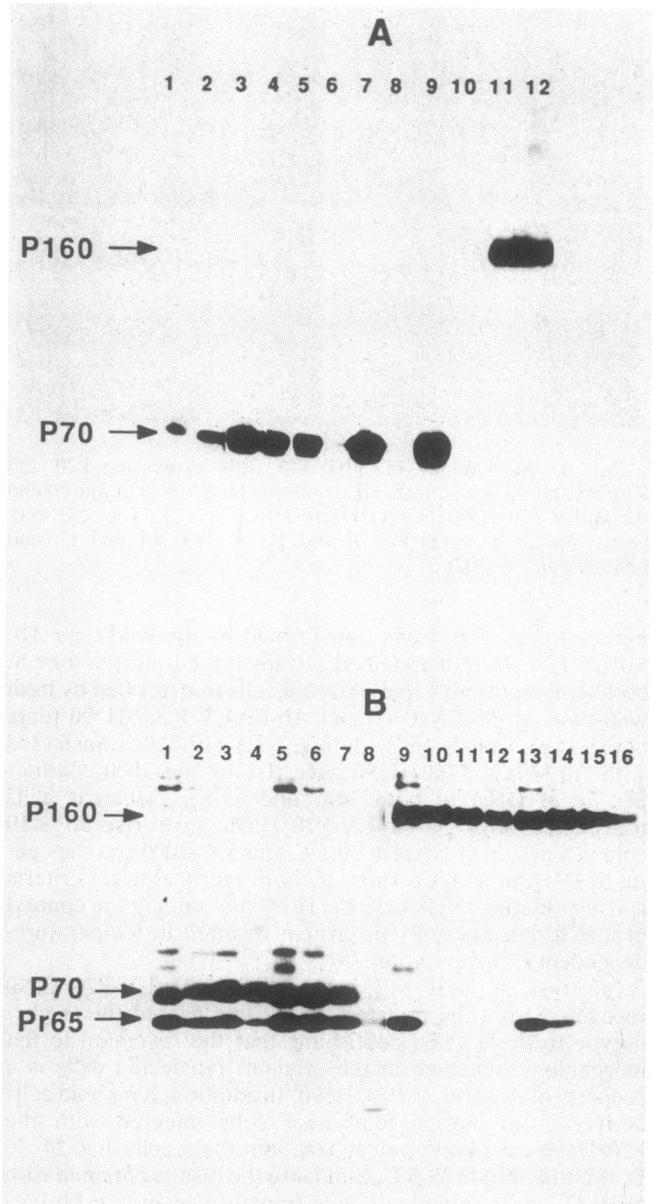

FIG. 5. Expression of P70/H590 in eukaryotic cells. (*A*) Extracts from NIH 3T3 cells expressing proteins P70 (lanes 1–4) and P70/H590 (lanes 5–8) and lymphoid cells expressing proteins P70/H590 (lanes 9, 10) and P160/H590 (lanes 11, 12) were analyzed using the *in vitro* kinase assay (33). Odd-numbered lanes show extracts prepared from cells grown overnight at 34°C; even-numbered lanes show extracts from cells grown overnight at 39.5°C. The gel was exposed to Kodak XAR-4 film for 2 hr. (*B*) Synthesis of P70/H590 and P160/H590. Lymphoid cells transformed with Ab-MuLV-P70/H590 (lanes 1–8) and Ab-MuLV-P160/H590 (lanes 9–16) were grown at 34°C (lanes 1–4 and 9–12) and 39.5°C (lanes 5–8 and 13–16) for 18 hr. Cells were labeled with [^{35}S]methionine (200 μCi/ml) for 10 min. After removing a sample for immediate processing (lanes 1, 5, 9, and 13), cells were resuspended in fresh medium and incubated for 1 (lanes 2, 6, 10, and 14), 2 (lanes 3, 7, 11, and 15), and 4 hr (lanes 4, 8, 12, and 16). Extracts, standardized for the amount of radioactivity, were immunoprecipitated and analyzed in a 10% polyacrylamide gel. The gel was exposed to Kodak XAR-4 film for 96 (lanes 1–8) and 48 hr (lanes 9–16).

and 14–16). This may reflect the high temperature used as a nonpermissive condition or may reflect the presence of the H590 mutation. The half-life of the P70/H590 protein is reduced as compared with that of the P160/H590 protein at the nonpermissive temperature (compare lanes 7 and 8 with lanes 15 and 16; data not shown). However, this effect cannot account for the dramatic reduction in protein kinase activity recovered from cells grown at the nonpermissive temperature. Thus, the loss of protein kinase activity at the nonpermissive temperature is probably responsible for the conditional phenotype of Ab-MuLV-P70/H590-transformed cells.

DISCUSSION

We have engineered ts Ab-MuLV mutants using sequence information from a functionally related protein, pp60 v-*src*. Expression of both of these mutations in a bacterial system results in reduced phosphorylation at high temperature. In addition, an Ab-MuLV strain carrying one of the mutations, H590, results in ts *in vitro* transformation of both fibroblasts and lymphoid cells. Preliminary results indicate that Ab-MuLV-P70/G536, but not Ab-MuLV-P120/G536, induces temperature-dependent transformation of NIH 3T3 cells (unpublished data). The fact that these mutations result in conditional transformation only when expressed in the context of the P70 protein is consistent with the idea that the long carboxyl-terminal portion of the Ab-MuLV protein is important in regulating the activity of P160 (39). Furthermore, these data indicate that suppression of a ts mutation can be conferred by domains of the protein distant from the lesion itself.

At the time this work was initiated, no ts strains of Ab-MuLV were known. Very recently, two other groups (16, 17) have isolated conditional Ab-MuLV strains. In the one case (17), biological assays relying on changes in the differentiation state of lymphoid transformants were used to identify the mutant. At least some lymphoid cells transformed by this virus undergo differentiation-like changes when maintained at the restrictive temperature. Sequence analysis of this isolate has not been reported, so it is difficult to compare the change(s) present in this virus with those we have studied. However, preliminary experiments using Ab-MuLV-P70/H590 suggest a similar phenotype in that some transformed clones derived from fetal liver cells express cytoplasmic μ protein after shift to the nonpermissive temperature (unpublished results).

Kipreos *et al.* (16) have used linker insertion mutagenesis to generate conditional Ab-MuLV transformation mutants. In their work, addition of a methionine residue between amino acids 448 and 449 or a valine-proline dipeptide between amino acids 460 and 461 gave rise to a ts mutant (16). Interestingly, these mutations are in the same region of the *abl* protein as the three-residue deletion prominent in a ts RSV strain examined by Nishizawa *et al.* (40) but lie considerably more toward the amino terminal than the mutations examined in this study. Similar to the mutations we have studied, these changes do not alter amino acids that are conserved in all protein-tyrosine kinases.

The strategy used to generate the G536 mutation involved simple substitution of a neutral residue for a charged residue. In contrast, the approach used for the H590 virus involved a more complex sequence analysis. In this case, expression of pp60 in which glycine 478 (Fig. 6) was replaced by aspartic acid resulted in ts transformation. A second substitution of tyrosine for histidine at position 492 restored wild-type function. The ability to isolate a ts Ab-MuLV by substituting histidine for tyrosine at the position homologous to residue 492 of pp60 indicates that the tyrosine at this position in Abelson protein masks the effect of the aspartic acid at position 576 (the equivalent of position 478 in pp60). The presence of a tyrosine residue at the position homologous to residue 492 of pp60 may be important for protein function. With the exception of pp60 and the products of the closely related *yes*, *fyn*, and *syn* genes, most other family members contain a tyrosine residue at this position (Fig. 6). This fact, coupled with the observation that substitution of tyrosine for

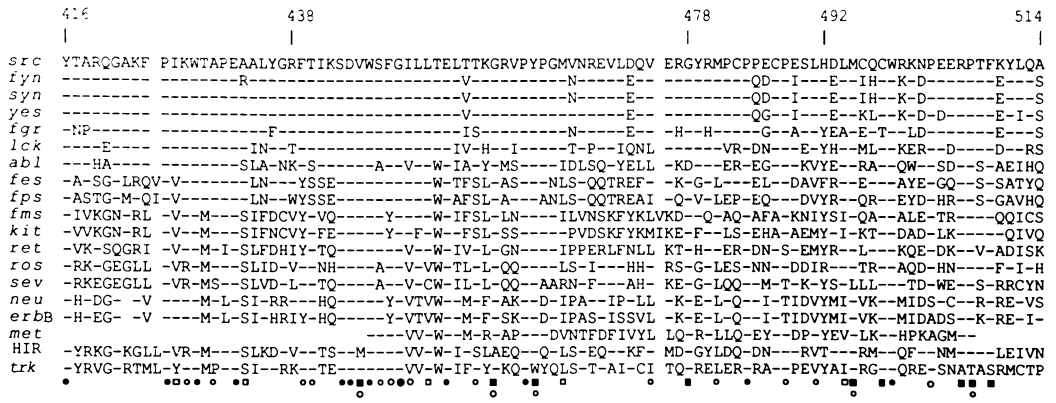

FIG. 6. Homology among protein-tyrosine kinases. Predicted amino acid sequences of oncogene products (35, 36, 41–51) were manually aligned for maximum homology. Residues identical to those in protein pp60 *src* are indicated by (-). Numbers refer to pp60 residues. Positions at which all residues are conserved (●), all residues are conserved but one (■), all substitutions are conservative (○), and all substitutions are conservative but one (□) are indicated below the sequences.

histidine 492 is implicated in reversion of another ts RSV strain, *tsLA24* (35), is consistent with this idea. Thus, changing the tyrosine to histidine in other protein-tyrosine kinases, or changing this residue in combination with the residue corresponding to position 478 of protein pp60 may yield other ts proteins.

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