Conservation of Function of *Drosophila melanogaster abl* and Murine *v-abl* Proteins in Transformation of Mammalian Cells

GINA D. HOLLAND, MARK J. HENKEMEYER, DONAL A. KAEHLER, F. MICHAEL HOFFMANN,
AND REX RISSE*

McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, Wisconsin 53706

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The *Drosophila melanogaster abl* and the murine *v-abl* genes encode tyrosine protein kinases (TPKs) whose amino acid sequences are highly conserved. To assess functional conservation between the two gene products, we constructed *Drosophila abl/v-abl*-chimeric Abelson murine leukemia viruses. In these chimeric Abelson murine leukemia viruses, the TPK and carboxy-terminal regions of *v-abl* were replaced with the corresponding regions of *D. melanogaster abl*. The chimeric Abelson murine leukemia viruses were able to mediate morphological and oncogenic transformation of NIH 3T3 cells and were able to abrogate the interleukin-3 dependence of a lymphoid cell line. We also found that a virus that contained both TPK and carboxy-terminal *Drosophila abl* regions had no in vitro transforming activity for primary bone marrow cells and lacked the ability to induce tumors in susceptible mice. A virus that replaced only a portion of the *v-abl* TPK region with that of *Drosophila abl* had low activity in in vitro bone marrow transformation and tumorigenesis assays. These results indicate that the transforming functions of *abl* TPKs are only partially conserved through evolution. These results also imply that the TPK region of *v-abl* is a major determinant of its efficient lymphoid cell-transforming activity.

The *abl* proto-oncogene has been conserved through evolution from invertebrates to vertebrates. *abl* gene homologs have been found in the genomes of such diverse species as *Caenorhabditis elegans* (13), *Drosophila melanogaster* (24, 55), mice (3, 14, 64), cats (52), and humans (20, 57, 64). In mice (1, 2, 14), cats (4-6), and humans (18, 20, 23), alterations in the structure and expression of *abl* proto-oncogenes give rise to neoplastic disease. In *D. melanogaster*, homozygous loss of function mutations in the *abl* proto-oncogene results in abnormal development (12, 22).

Retroviral transduction of murine *c-abl* gene sequences by Moloney murine leukemia virus (M-MuLV) resulted in the formation of the oncogenically activated *v-abl* gene of Abelson murine leukemia virus (A-MuLV) (1, 2, 14). The *v-abl* gene, which fuses the first 236 M-MuLV *gag*-encoded amino acids to 1,219 carboxy-terminal *c-abl*-encoded amino acids (33, 44, 56, 64), is required for the fibroblast- and lymphoid cell-transforming activities of A-MuLV (42, 43, 60, 65). Intravenous or intraperitoneal injection of mice with A-MuLV usually induces pre-B-cell tumors (16, 41, 58).

The *Drosophila abl* gene encodes a protein of 1,521 amino acids which has tyrosine protein kinase (TPK) activity when expressed in bacteria (21). Homozygous loss of function mutations in the *Drosophila abl* gene are usually lethal by the pharate adult stage of development (22). Adult *abl* mutant flies are lethargic, show reduced viability and fecundity, and have roughened eyes (22).

Analysis of the deduced amino acid sequence of the *D. melanogaster abl* gene-coding region shows that it shares significant identity with the *v-abl* oncogene and with the human and murine *c-abl* proto-oncogenes (21). The proteins encoded by the *v-abl*, human and murine *c-abl*, and *Drosophila abl* genes show the most amino acid identity in the *src*-homology 2 (SH2) region (9, 36, 51, 61) (86% similarity and 78% identity) and in the TPK region (87% similarity and 77% identity). In addition, the *Drosophila abl* and murine

and human *c-abl* genes are 60% identical in the *src*-homology 3 (SH3) (36, 61) region, which is located 5' to the SH2 region. Little sequence identity is seen in the carboxy portions of these molecules (28% similarity and 16% identity).

Overexpression of the normal *c-abl* gene does not result in cell transformation (26, 63). Instead, oncogenic activation of the murine and human *c-abl* genes requires the presence of an amino-terminal myristoylation site and deletion of negative regulatory sequences 5' to the SH2 region (11, 26, 63). The myristoylation site can be supplied by either *c-abl* sequences or M-MuLV *gag* sequences (11, 26, 63).

In light of the high degree of amino acid sequence conservation between the *D. melanogaster* and *v-abl* proteins, it is of interest to determine whether evolutionary conservation of sequence implies functional conservation. Accordingly, we sought to determine whether (i) the transforming potential of the *Drosophila abl* gene can be activated by the same types of lesions shown to activate the transforming potentials of the murine and human *c-abl* genes; (ii) an activated *Drosophila abl* gene can substitute for the *v-abl* gene in A-MuLV; and (iii) the catalytic region of *Drosophila abl*, the TPK region, can substitute for the *v-abl* TPK region. Therefore, we constructed chimeric A-MuLV proviruses that substitute the TPK and carboxy-terminal regions of *v-abl* with the corresponding regions of *Drosophila abl*. We then analyzed the chimeric *Drosophila abl/v-abl* proviruses for their fibroblast- and lymphoid cell-transforming activities.

MATERIALS AND METHODS

Cell lines and viruses. The NIH 3T3 cell line (27) used in transfection and virus infection experiments was cultured in Dulbecco modified Eagle medium supplemented with 3 to 5% calf serum (Hyclone, Logan, Utah). The interleukin 3 (IL-3)-dependent lymphoid cell line, FL5.12 (38), was supplied by J. McCubrey, East Carolina University, Greenville, N.C., and was cultured in Iscove modified Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone) and 5% WEHI-3 conditioned medium as a

* Corresponding author.

FIG. 1. Chimeric A-MuLVs. Proviral structures of pABpro, pA-MuLV(cD), pA-MuLV(D), and pA-MuLV(sd). Restriction endonuclease sites used for cloning and recombination steps are indicated. Solid lines, M-MuLV non-coding sequences; open boxes, A-MuLV long terminal repeats; cross-hatched boxes, A-MuLV *gag* sequences; black boxes, *v-abl* sequences; hatched boxes *D. melanogaster abl* sequences. ATP-B, ATP-binding site; SH2, *src*-homology 2 region; SH3, *src*-homology 3 region; TPK, tyrosine protein kinase region; Tyr-P, site of tyrosine autophosphorylation. 1, 2, 3, and p4C1, DNA probes used for Southern hybridization studies.

source of IL-3 (25). Primary bone marrow cells were collected from the tibias and femurs of 4- to 6-week-old BALB/c mice and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 μ M β -mercaptoethanol. All culture media were also supplemented with 100 U of penicillin, 100 μ g of streptomycin, and 100 μ g of sodium pyruvate (GIBCO Laboratories, Grand Island, N.Y.) per ml. All cells were cultured in humidified incubators at 37°C with 5% CO₂. NIH 3T3 cells were transfected by the calcium phosphate precipitation method as described before (16). For virus production, 2 μ g of construct DNA [A-MuLV(cD), A-MuLV(D), or A-MuLV(sd)] and 2 μ g of pMOV-3 M-MuLV proviral clone (19) DNA were cotransfected into NIH 3T3 cells. Titers of virus stocks derived from morphologically transformed cell lines were determined by focus formation on NIH 3T3 cells (53) and by UV-XC plaque formation (50). FL5.12 cells were infected with virus and assayed for factor abrogation as described previously (37). Twenty-four hours after infection, 10⁶ cells were exposed to 4,000 rads in a ¹³⁷Cs irradiator (J. L. Shepard and Associates, Glendale, Calif.) and plated as infectious centers as described before (37). Infection of bone marrow cells was

performed in liquid culture (16, 48). Following infection, the cells were plated in either 96-well microdilution plates (Falcon; Becton Dickinson Labware, Lincoln Park, N.J.) at a density of 5 $\times$ 10⁵ cells per ml (mass culture assay) (48) or 0.35% agarose-containing medium at a density of 2 $\times$ 10⁶ cells per 60-mm dish (Falcon; Becton-Dickinson Labware) over a layer of 0.7% agarose-containing medium (agarose transformation assay), as described before (16, 47).

Constructs and DNA analysis. The A-MuLV(cD) construct (Fig. 1) was prepared by inserting the 5,297-base-pair (bp) *NotI*-*BglII* fragment of a *Drosophila abl* gene cDNA (21) into an *EagI*-*BglII*-cleaved A-MuLV proviral clone, pABpro (16). The *NotI* site was introduced into the *Drosophila abl* cDNA by addition of *NotI* linkers (New England BioLabs, Inc., Beverly, Mass.) at the unique *FspI* site. A-MuLV(cD) is expected to encode a protein of 180 kilodaltons which is derived exclusively from the *Drosophila abl* sequences. The A-MuLV(D) construct (Fig. 1) was prepared by inserting the 4,085-bp *NcoI*-*BglII* fragment of the *Drosophila abl* gene cDNA into the *NcoI*-*BglII*-cleaved pABpro. The *NcoI*-*BglII* fragment of the *Drosophila abl* gene cDNA encodes 209 amino acids of the TPK region and 1,153 amino acids of the

carboxy portion of the *Drosophila abl* protein. The A-MuLV(D) construct therefore fuses 1,362 fly *abl*-encoded amino acids to the 236 *gag*-encoded amino acids and the 161 *v-abl*-specific amino acids contained in the amino-terminal portion of *v-abl*. The resulting chimeric protein, *gag-abl*(D), will contain a chimeric TPK region with the ATP-binding site (28) derived from *v-abl* and the tyrosine phosphorylation site (30, 31) derived from *Drosophila abl*. The *gag-abl*(D) protein is predicted to have a molecular size of 180 kilodaltons. The A-MuLV(sd) construct (Fig. 1) was prepared by inserting a 665-bp *NcoI-HinI* fragment of the *Drosophila abl* gene cDNA into *NcoI-HinI*-cleaved pABpro. The A-MuLV(sd) construct encodes the 397 amino-terminal amino acids of *v-abl* fused to 209 amino acids of the *Drosophila abl* TPK region plus 12 non-TPK *Drosophila abl* amino acids, with the remainder of the chimeric protein being derived from *v-abl*. Like the *gag-abl*(D) protein, the *gag-abl*(sd) protein contains a chimeric TPK domain whose ATP-binding site derives from *v-abl* and whose tyrosine phosphorylation site derives from *Drosophila abl*. Both the A-MuLV(D) and A-MuLV(sd) constructs contain the *v-abl* SH2 region (9, 51).

As all restriction endonuclease sites used for the construction of A-MuLV(D) and A-MuLV(sd) are conserved between the *v-abl* and *Drosophila abl* genes, our strategy for creating recombinant *abl* genes does not result in the alteration of the sequence of either substrate *abl* gene.

DNA sequence analysis. The 680-bp *HincII-BglII* and 534-bp *BglII-PstI* fragments of the *v-abl* gene of pABpro (16), which together encompass the entire TPK region of *v-abl*, were molecularly cloned into the sequencing vectors M13mp18 and M13mp19. Single-stranded bacteriophage DNA was sequenced with the Sequenase Version 2.0 DNA sequencing kit under conditions supplied by the manufacturer (United States Biochemical Corp., Cleveland, Ohio). The M13 sequencing primer (New England BioLabs) was used to prime the sequencing reactions. The products of the sequencing reactions were resolved on 6% polyacrylamide gels containing 7 M urea and 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA [pH 8.0]).

DNA preparation and Southern blotting. High-molecular-weight DNA was isolated from cell lines and tumors as described before (16, 17). A 10-μg amount of DNA was cleaved to completion with restriction endonucleases under conditions supplied by the manufacturer (New England BioLabs). The DNA fragments were then resolved by electrophoresis through 0.7% agarose gels, transferred to nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) (59), and hybridized to random-primer-labeled probes (10). Probes were labeled with [α - 32 P]dCTP, ~3,000 Ci/mmol (Amersham Corp., Arlington Heights, Ill.), under conditions supplied by the manufacturer of the random-primer-labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Nucleic acid probes. Probe 1 was a 473-bp *HincII-NcoI* fragment of *v-abl*, probe 2 was a 660-bp *XhoI-SalI* fragment of *v-abl*, and probe 3 was a 1.2-kilobase (kb) *NcoI* fragment of *v-abl* (Fig. 1). The *Drosophila abl*-specific p4C1 probe has been described previously (Fig. 1) (21).

Metabolic labeling, immunoprecipitation, and immune complex kinase assay. Cells were labeled with [35 S]-methionine, >800 Ci/mmol (Amersham Corp.); cell lysates were prepared and immunoprecipitations were performed as described previously (15, 29). In vitro kinase assays were performed as described before (15, 62; J. Y.-J. Wang, personal communication). Equal amounts of protein, as assayed by the Bradford assay, were used in the in vitro

kinase reactions (7). [γ - 32 P]ATP, ~3,000 Ci/mmol (Amersham Corp.), was used as the phosphate donor in the in vitro kinase experiments. Products of the immunoprecipitation and in vitro kinase assays were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (32). Gels (7.5 or 10% polyacrylamide-SDS) were run at constant current. After electrophoresis, gels used for resolving in vitro kinase assay products were routinely treated with 1 M KOH at 55°C for 1 h to remove phosphoserine and phosphothreonine residues (Wang, personal communication).

Tumorigenesis assays. To assess oncogenic transformation of A-MuLV(D) or A-MuLV(sd)-transformed NIH 3T3 cell lines, 10^6 cells were injected subcutaneously into the backs of 4- to 8-week-old BALB/c nude mice. The animals were then monitored biweekly for tumor formation. For assaying the tumorigenicity of A-MuLV(D) or A-MuLV(sd) viruses, BALB/cJ weanling mice were injected intravenously via the retro-orbital sinus with 1.5×10^4 focus-forming units (FFU) of virus. The mice were observed triweekly for tumor formation and sacrificed when moribund. All mice were obtained from either our colonies or the University of Wisconsin Gnotobiote Laboratories, Madison.

RESULTS

Morphological transformation of NIH 3T3 cells mediated by *Drosophila abl/v-abl*-chimeric A-MuLVs. To test the abilities of A-MuLV(cD), A-MuLV(D), and A-MuLV(sd) (Fig. 1) to transform murine fibroblastoid cells morphologically, these constructs were transfected with a molecularly cloned helper virus, pMOV-3 (19), into NIH 3T3 cells. We found that both A-MuLV(D) and A-MuLV(sd) proviral clones morphologically transformed NIH 3T3 cells, although with approximately 10-fold-lower efficiency than the parent A-MuLV proviral clone, pABpro (16). The A-MuLV(cD) proviral clone did not transform NIH 3T3 cells. The transformation efficiencies, averaged over three experiments with three different plasmid preparations of each clone, were 2.4×10^2 FFU/μg for pA-MuLV(D) and 1.3×10^2 FFU/μg for pA-MuLV(sd). The average transformation efficiency for pABpro in these experiments was 1.6×10^3 FFU/μg. NIH 3T3 cells transformed by A-MuLV(D) or A-MuLV(sd) exhibited a rounded refractile morphology similar to that of A-MuLV-transformed cells (53). The A-MuLV(D)- and A-MuLV(sd)-induced foci did not grow as rapidly as foci induced by A-MuLV, and the cells frequently detached from the plate (data not shown). Virus stocks produced from A-MuLV(D)- and A-MuLV(sd)-transformed cell lines induced morphological transformation of NIH 3T3 cells similar to that observed following transfection of these constructs (data not shown). Virus pools from A-MuLV(D)- and A-MuLV(sd)-transformed cell lines routinely yielded titers of 1×10^4 to 3×10^4 FFU/ml, which are somewhat lower than the titer of pools from wild-type A-MuLV-transformed cell lines.

Analysis of A-MuLV(D) and A-MuLV(sd) integrated proviruses. To determine whether the A-MuLV(D) and A-MuLV(sd) proviruses had the expected structures following transfection, genomic DNA was extracted from A-MuLV(D)- and A-MuLV(sd)-transformed cell lines and was analyzed by Southern blotting, using probes directed to both the 5' and 3' regions of *v-abl* and to *Drosophila abl* sequences (Fig. 1). Hybridization of *XbaI*-cleaved A-MuLV and A-MuLV(D) cell line DNAs with probe 1 showed genome size fragments of 5.8 and 6.7 kb, respectively, as predicted (Fig. 2, probe 1 panel, lanes 2 and 3). To confirm

FIG. 2. Southern blot analysis of DNAs of A-MuLV(D)-transfected cells. DNAs (10 μ g) were digested with *Xba*I and electrophoresed through a 0.7% agarose gel. After transfer, the blots were hybridized to the probes indicated above each panel. Lanes: 1, NIH 3T3 cells; 2, an A-MuLV(D)-transformed cell line; 3, an A-MuLV-transformed cell line. The positions of the genome size 6.7-kb A-MuLV(D) fragment, the genome size 5.8-kb A-MuLV fragment, and the 11.0-kb endogenous *c-abl* fragment are indicated by arrows.

further the structure of the integrated A-MuLV(D) provirus, Southern blot analysis with probe 2 was performed. Hybridization of *Xba*I-cleaved A-MuLV and A-MuLV(D) cell line DNAs with probe 2 (Fig. 1) showed a genome size 5.8-kb fragment only in the A-MuLV cell line DNA, as predicted (data not shown). Finally, to demonstrate that *Drosophila abl* gene sequences were present in the integrated A-MuLV(D) provirus, Southern blot analysis of *Xba*I-cleaved A-MuLV and A-MuLV(D) cell line DNAs was performed by using the *Drosophila abl*-specific probe p4C1 (21) (Fig. 1). The p4C1 probe detected a 6.7-kb *Xba*I fragment only in the DNA of the A-MuLV(D) cell line (Fig. 2, p4C1 panel, lane 2).

Because the A-MuLV(sd) construct was expected to yield the same size *Xba*I fragment as the parent A-MuLV, Southern blot analysis with different restriction endonucleases and probes was performed on the DNA of an A-MuLV(sd)-transformed cell line. Hybridization of *Nco*I-cleaved A-MuLV and A-MuLV(sd) cell line DNAs with probe 3 (Fig. 1) showed 1.2- and 1.4-kb fragments, respectively, as predicted (Fig. 3, probe 3 panel, lanes 2 and 3). To confirm that *Drosophila abl* sequences were present in the 1.4-kb *Nco*I fragment from A-MuLV(sd) cells, the p4C1 probe was used in the Southern hybridization experiments. The 1.4-kb *Nco*I fragment was detected by the p4C1 probe only in A-MuLV(sd) cells (Fig. 3, p4C1 panel, lane 2).

These analyses indicate that the A-MuLV(D) and A-MuLV(sd) integrated proviruses had the expected structures, as depicted (Fig. 1), and that these constructs did not undergo gross deletions or rearrangements upon transfection and transmission.

Expression of A-MuLV(D) and A-MuLV(sd) chimeric proteins in transformed fibroblasts. To determine whether chimeric *gag* fusion proteins of the expected sizes were synthesized in A-MuLV(D)- and A-MuLV(sd)-transformed NIH 3T3 cells, A-MuLV(D) and A-MuLV(sd) cell lines were metabolically labeled with [35 S]methionine, and cell lysates were prepared. These cell lysates were then immunoprecipitated by using anti-p15^{gag} serum. This serum binds the *v-abl* protein, p160^{v-abl}, as well as the M-MuLV structural protein precursor, pr65^{gag}, and would be expected to bind a 180-kilodalton A-MuLV(D)-encoded protein and a 160-kilodalton

FIG. 3. Southern blot analysis of A-MuLV(sd)-transfected cells. DNAs (10 μ g) were digested with *Nco*I and electrophoresed through a 0.7% agarose gel. After transfer, the blots were hybridized to the probes indicated above each panel. Lanes: 1, NIH 3T3 cells; 2, an A-MuLV(sd)-transformed cell line; 3, an A-MuLV-transformed cell line. The positions of the 1.4-kb A-MuLV(sd)-specific fragment, the 1.2-kb A-MuLV-specific fragment, and the 5.3-, 2.4-, and 0.8-kb endogenous *c-abl* fragments are indicated by arrows.

A-MuLV(sd)-encoded protein. SDS-polyacrylamide gel electrophoresis analysis of the immunoprecipitates showed that the A-MuLV(D) and A-MuLV(sd) cell lines produced *gag*-related proteins of the predicted sizes of 180 and 160 kilodaltons, respectively (Fig. 4, lanes 5 to 8). In addition, the M-MuLV-encoded *gag* precursor protein, pr65^{gag}, was also synthesized by the A-MuLV(D) and A-MuLV(sd) cell lines (Fig. 4, lanes 5 to 8).

Because activated *abl* proteins have the ability to phosphorylate themselves and exogenous substrates in immune precipitates (35, 42, 43, 54, 66), the *in vitro* kinase activities of the p180^{gag-abl(D)} and p160^{gag-abl(sd)} proteins were analyzed. Unlabeled lysates prepared from the A-MuLV(D) and A-MuLV(sd) cell lines were immunoprecipitated, and the immune complexes were assayed for *in vitro* kinase activity. Both p180^{gag-abl(D)} and p160^{gag-abl(sd)} were capable of autophosphorylation (Fig. 5). The additional bands seen in the A-MuLV(D), A-MuLV(sd), and A-MuLV lanes are of unknown origin and may represent either cellular proteins that form complexes with the *abl* proteins or proteolytic degradation products of the *abl* proteins. Experiments to determine the nature of the proteins in these additional bands are in progress. Both the *gag-abl(D)* and *gag-abl(sd)* proteins exhibited a preference for Mn²⁺ over Mg²⁺ for autophosphorylation, in common with the *v-abl* protein (data not shown). Also, both of the chimeric *v-abl* proteins were able to phosphorylate the exogenous substrate, α -casein (data not shown). Thus, the proteins encoded by A-MuLV(D) and A-MuLV(sd) were active in the *in vitro* kinase assay in a manner similar to the *v-abl* protein.

Tumorigenicity of A-MuLV(D)- and A-MuLV(sd)-trans-

FIG. 4. Protein characterization of A-MuLV(D)- and A-MuLV(sd)-transformed cell lines. Cells were labeled with [35 S]methionine for 3 h at 37°C. Some 5×10^4 cell equivalents of the labeled cell extracts were immunoprecipitated with an α -p15^{gag} antiserum, and immune complexes were resolved by electrophoresis through a 10% polyacrylamide-SDS gel (lanes 1 to 6) or a 7.5% polyacrylamide-SDS gel (lanes 7 and 8). In lanes 2, 4, 6, and 8, the α -p15^{gag} serum used for immunoprecipitation was preincubated with unlabeled M-MuLV proteins. Lanes: 1 and 2, NIH 3T3 cells; 3 and 4, an A-MuLV-transformed cell line; 5 and 6, an A-MuLV(D)-transformed cell line; 7 and 8, an A-MuLV(sd)-transformed cell line. The positions of the p160^{v-abl}, p180^{gag-abl(D)}, p160^{gag-abl(sd)}, and pr65^{gag} proteins are indicated by arrows.

formed fibroblasts. In addition to morphologically transforming murine fibroblastoid cell lines, e.g., NIH 3T3, A-MuLV can also oncogenically transform these cells. To determine whether the morphologically transformed A-MuLV(D) and A-MuLV(sd) cell lines were capable of growing as tumors, BALB/c nude mice were injected subcutaneously with 10^6 cells and observed for tumor formation. We observed that A-MuLV(D) and A-MuLV(sd) cells, in common with A-MuLV cells, formed progressively growing tumors at the site of injection (Table 1). While the latent period for tumor development by the A-MuLV(D) cells was markedly longer than that observed for A-MuLV cells cultured for an equivalent period of time, the latent period for tumor development by A-MuLV(sd) cells was similar to that of the A-MuLV cells (Table 1).

Hybridization of *Xba*I-cleaved DNAs of the A-MuLV(D) and A-MuLV(sd) cell line-induced tumors with the p4C1

probe showed *Drosophila abl*-hybridizing fragments of either 6.7 [A-MuLV(D)] or 5.8 [A-MuLV(sd)] kb as predicted (data not shown). Hybridization of *Bgl*II-cleaved DNAs from these tumors with the p4C1 probe showed that the tumor DNAs had the same pattern of proviral integration as the inoculated cell lines (data not shown).

In vitro lymphoid cell-transforming activities of A-MuLV(D) and A-MuLV(sd) viruses. In addition to the ability to transform murine fibroblastoid cell lines morphologically and oncogenically, activated *abl* genes can effect the in vitro transformation of primary lymphoid cells (48). *v-abl* is unique among the members of the *src* family of TPKs in its ability to efficiently transform primary lymphoid cells. We therefore assayed the A-MuLV(D) and A-MuLV(sd) viruses for their abilities to transform bone marrow lymphoid cells in the mass culture (48) and agarose colony (47) assays. Although we observed extensive colony formation following infection of bone marrow cells with A-MuLV, infection of bone marrow cells with A-MuLV(D) did not give rise to agarose colonies (Table 2). Infection of bone marrow cells with A-MuLV(sd) did give rise to agarose colonies, but with 25-fold-lower efficiency than A-MuLV (Table 2). As the agarose colony assay is perhaps the most stringent test of A-MuLV lymphoid cell-transforming ability, we also assayed A-MuLV(D) and A-MuLV(sd) viruses for the ability to transform bone marrow cells in the mass culture assay. Again, although infection of bone marrow cells with A-MuLV gave rise to transformed cultures, infection of bone marrow cells with A-MuLV(D) did not give rise to trans-

FIG. 5. In vitro kinase activity of A-MuLV(D)- and A-MuLV(sd)-encoded proteins. Protein content of cell lysates was assayed by using the Bradford assay (7), and equivalent amounts of protein were immunoprecipitated with α -p15^{gag} antiserum. Immune complexes were washed and suspended in Mn^{2+} -kinase reaction buffer containing 1 μ Ci of [γ - 32 P]ATP. The reaction mixtures were incubated at 30°C for 15 min, after which the immune complexes were washed in kinase lysis buffer and suspended in Laemli sample buffer. Samples were electrophoresed through 7.5% polyacrylamide-SDS gels, and the gel was treated with 1 M KOH for 1 h at 55°C to remove phosphoserine and phosphothreonine residues. Phosphoproteins were visualized after autoradiography of the dried gel. Lanes: 1, NIH 3T3 cells; 2, an A-MuLV(sd)-transformed cell line; 3, an A-MuLV(D)-transformed cell line; 4, an A-MuLV-transformed cell line. The positions of the p160^{v-abl}, p180^{gag-abl(D)}, and p160^{gag-abl(sd)} proteins are indicated by arrows.

TABLE 1. Tumorigenicity of chimeric A-MuLV-transformed NIH 3T3 cell lines in nude mice

Cell line ^a	No. of mice	No. of tumors	Latency (days)	Provirus ⁺ ^a
NIH 3T3	2	0	>90	
A-MuLV 1	2	2	10-16	ND ^b
A-MuLV(D) 1	4	4	28-45	4/4
A-MuLV(sd) 1	3	3	15-25	3/3

^a Tumor DNAs were cleaved with *Xba*I and were examined by Southern blotting, using the p4C1 probe, for the presence of genome size [6.7-kb A-MuLV (D) or 5.8-kb A-MuLV (sd)] *Drosophila abl*-hybridizing fragments.

^b ND, Not done.

TABLE 2. Transformation of lymphoid cells by chimeric A-MuLVs

Virus	Input virus titer (FFU)	Agarose ^a		Mass culture (positive cultures/total cultures) ^b
		No. of colonies	No. of plates	
M-MuLV	2.0 × 10 ⁶ XC PFU	0	12	0/18
A-MuLV	1.5 × 10 ⁴	53.8 ± 35.9	12	15/15
	1.5 × 10 ³	3.4 ± 1.2	4	12/17
A-MuLV(D)	1.5 × 10 ⁴	0.3 ± 0.9	26	0/32
A-MuLV(sd)	1.5 × 10 ⁴	2.3 ± 2.5 ^d	12	3/34 ^c

^a Agarose transformation assays were performed as described in Materials and Methods. The mean number of agarose colonies per 2 × 10⁶ cells plated was scored at 14 days postinfection.

^b Cultures were initially plated at 5 × 10⁵ bone marrow cells per ml as described in Materials and Methods and scored for the presence of proliferating lymphocytes after 1 month.

^c Cells from agarose colonies or mass cultures of A-MuLV(sd)-infected cells could rarely be propagated beyond 4 to 6 weeks postinfection. The diameter of agarose colonies induced by A-MuLV(sd) was 30 to 50% of the diameter of agarose colonies induced by A-MuLV.

formed cultures (Table 2). Proliferation of bone marrow cells infected with A-MuLV(sd) was observed for a period of 1 to 2 weeks, after which the cultures usually degenerated (Table 2). While 5 of 7 (71.4%) A-MuLV-transformed cultures gave rise to cell lines, only 1 of 24 (4.2%) A-MuLV(sd)-transformed cultures could be continuously propagated. Southern blot analysis, using the p4C1 probe, of the DNA of this A-MuLV(sd) cell line showed that the DNA contained a 5.8-kb genome size *Xba*I fragment and the A-MuLV(sd)-specific 1.4-kb *Nco*I fragment (data not shown). Thus, while A-MuLV(D) was incapable of transforming bone marrow cells, A-MuLV(sd) had residual lymphoid cell-transforming activity.

Abrogation of IL-3 dependence in hemopoietic cell lines by A-MuLV(D) and A-MuLV(sd) viruses. Another aspect of the transforming activity of activated *abl* genes is the ability to abrogate efficiently the IL-3 dependence of IL-3-dependent murine hemopoietic cell lines (8, 39, 40). As the A-MuLV(D) virus did not transform bone marrow cells in vitro, it was of particular interest to determine whether the chimeric A-MuLVs could abrogate the IL-3 dependence of hemopoietic cell lines. We infected the IL-3-dependent lymphoid cell line FL5.12 (38) with A-MuLV(D) or A-MuLV(sd) and selected directly for factor independence. We observed that infection of the FL5.12 cell line with A-MuLV(D) or A-MuLV(sd) viruses could induce the development of IL-3-independent colonies (Table 3), although with lower frequencies than A-MuLV. Southern blot analysis, using the p4C1 probe, of *Xba*I-cleaved DNAs of the IL-3-independent cell lines

showed the presence of either a genome size 6.7-kb A-MuLV(D) or a genome size 5.8-kb A-MuLV(sd) fragment, as expected (data not shown). Therefore, although the A-MuLV(D) virus was not able to transform bone marrow cells in vitro, it was able to function in lymphoid cells as measured by its ability to abrogate the IL-3 dependence of the FL5.12 lymphoid cell line. These results make it unlikely that the A-MuLV(D) protein is inherently unstable in lymphoid cells.

Tumorigenicity of A-MuLV(D) and A-MuLV(sd) viruses. To test the ability of the A-MuLV(D) and A-MuLV(sd) viruses to induce neoplasia, we injected weanling BALB/c mice intravenously with these viruses. After a long latent period, 7 of 38 mice injected with A-MuLV(D) developed disease characterized by enlarged spleens and lymph nodes, as seen in A-MuLV or M-MuLV diseases (1, 2, 45, 46) (Table 4). Southern blot analysis showed that none of these tumors contained *Drosophila abl*-hybridizing sequences and were most likely of M-MuLV helper virus origin. Therefore, the A-MuLV(D) virus did not induce tumors in susceptible mice. In contrast, 1 of 13 mice injected with the A-MuLV(sd) virus developed disease characterized by hind limb paralysis and enlarged lymph nodes and spleen 46 days after injection (Table 4). The symptom of hind limb paralysis, coupled with enlarged lymph nodes and spleen, is characteristic of classical Abelson virus disease (45, 45). Southern blot analysis, using the p4C1 probe, of DNAs from the tumor tissues (lymph node and spleen) of this animal showed the presence of the A-MuLV(sd)-specific 1.4-kb *Nco*I fragment in these DNAs (data not shown). Additional Southern blot analysis of *Xba*I-cleaved DNAs of the tissues showed that the *Drosophila abl*-hybridizing sequences were present on a genome size 5.8-kb fragment (data not shown). Therefore, A-MuLV(sd) could induce disease in mice, although with much less efficacy than A-MuLV.

DISCUSSION

To address the question of functional conservation among *abl* genes, we constructed *Drosophila abl/v-abl* chimeric

TABLE 3. Ability of chimeric A-MuLVs to abrogate the IL-3 dependence of a lymphoid cell line^a

Virus	Efficiency of infection ^b	IL-3 ⁻ wells/total wells (%)	Provirus ^{+c}
None		0/464 (<0.2)	
M-MuLV	0.001	2/412 (0.5)	ND ^d
A-MuLV	0.001	105/472 (22.2)	5/5
A-MuLV(D)	0.002	13/338 (3.8)	6/6
A-MuLV(sd)	0.002	31/332 (9.3)	5/5

^a Virus pools were assayed for ability to abrogate IL-3 dependence in three independent experiments.

^b The efficiency of infection was calculated as the number of infectious centers per 5 × 10⁶ cells divided by the number of input infectious units per 5 × 10⁶ cells. The number of infectious centers was determined as described in Materials and Methods.

^c DNAs of IL-3-independent cell lines were cleaved with *Xba*I and were examined by Southern blotting, using the p4C1 probe, for the presence of genome size [6.7-kb A-MuLV(D) or 5.8-kb A-MuLV(sd)] *Drosophila abl*-hybridizing fragments.

^d ND, Not done.

TABLE 4. Tumorigenicity of chimeric A-MuLVs

Virus	No. of mice	No. of tumors	Mean latency (days)	Provirus ^{+a}
A-MuLV ^b	14	14	22	14/14
A-MuLV(D)	38	7	83	0/7
A-MuLV(sd)	13	1	46	1/1

^a Tumor DNAs were cleaved with *Xba*I and were examined by Southern blotting, using the p4C1 probe, for the presence of genome size [6.7-kb A-MuLV(D) or 5.8-kb A-MuLV(sd)] *Drosophila abl*-hybridizing fragments.

^b Data from Green et al. (15).

FIG. 6. Comparison of the deduced amino acid sequences of the *v-abl* and *D. melanogaster abl* TPK regions within the *NcoI-HinfI* fragments recombined to construct pA-MuLV(sd). The top line indicates the *v-abl* amino acid sequence, deduced from the nucleic acid sequence of the TPK region of the *v-abl* gene of the A-MuLV provirus clone pABpro (16) (see Materials and Methods). The bottom line indicates the *Drosophila* (*Dr.*) *abl* deduced amino acid sequence (21). Identical amino acids are indicated by a solid bar, and conservative amino acid changes are indicated by two dots. The conserved *NcoI*, *BalI*, and *HinfI* restriction endonuclease sites are indicated by arrows. The sites for tyrosine autophosphorylation are indicated by circled Y's. The site at which a point mutation in *v-abl* results in the change of phenylalanine to valine is indicated by a circled V. The carboxy boundaries of the TPK regions of the *v-abl* and *Drosophila abl* proteins are indicated by an arrow and "End TPK."

A-MuLVs. Using these recombinants, we have demonstrated that some of the molecular properties and biological activities of the *v-abl* protein are conserved between *Drosophila abl* and *v-abl*.

Our studies of the A-MuLV(cD) construct indicated that the normal *Drosophila abl* gene in the context of A-MuLV did not morphologically transform NIH 3T3 cells. Although we have no evidence that the *abl*(cd) protein was expressed, it is most likely that the A-MuLV(cD) construct failed to transform NIH 3T3 cells because its encoded protein was inactive due to the presence of amino-terminal negative regulatory elements (11, 26, 63).

Replacement of sequences encoding the amino terminus and SH3 region of *Drosophila abl* with A-MuLV *gag* sequences generated the chimeric A-MuLV, A-MuLV(D). Transfection of this proviral construct into NIH 3T3 cells resulted in the morphological transformation of these cells. These results indicate that the transforming potential of the *Drosophila abl* gene could be activated by the addition of M-MuLV *gag* sequences and by the removal of negative regulatory sequences 5' to the SH2 region. These changes are similar to those which have been shown to activate the transforming potential of other *c-abl* genes (11, 26, 63). Our studies of the A-MuLV(D) chimera showed that it could also abrogate the IL-3 dependence of a lymphoid cell line. However, the *v-abl* primary lymphoid cell transformation function was not conserved in A-MuLV(D) as this virus was unable to effect in vitro bone marrow transformation. The leukemogenic activity of A-MuLV was also not conserved in A-MuLV(D) as this virus did not induce tumors in the most

susceptible host, the weanling BALB/c mouse (45, 46). Thus, *Drosophila abl* could only partially substitute for *v-abl* in A-MuLV.

The inability of A-MuLV(D) to transform primary lymphoid cells can be ascribed to several factors. First, the *v-abl* and *Drosophila abl* gene sequences show considerable divergence in the regions encoding the carboxy-terminal portions of the proteins. It may be that these differences give rise to altered substrate specificity, altered kinase activity, or altered protein stability. Indeed, the work of several investigators has suggested that regions downstream of the kinase region of *v-abl* can act to enhance the in vitro lymphoid cell-transforming and tumorigenic abilities of A-MuLV (49).

Second, examination of the TPK regions of *Drosophila abl* and *v-abl* reveals several nonconservative amino acid differences (Fig. 6). In particular, the *v-abl* gene sequenced here, as well as another *v-abl* gene (P. Jackson and D. Baltimore, unpublished results), encodes a valine residue at position 522 immediately downstream of the major site of tyrosine autophosphorylation (Fig. 6). In contrast, the *Drosophila abl* and human *c-abl* genes encode a phenylalanine residue at the corresponding position (Fig. 6). This change from phenylalanine to valine may contribute to the transforming activity of the *v-abl* protein.

To determine whether the inability of A-MuLV(D) to mediate transformation of bone marrow cells resulted from substitution of the TPK or carboxy-terminal region of *v-abl*, we constructed the A-MuLV(sd) chimera. This construct replaced only a portion of the TPK region of *v-abl* with that

of *Drosophila abl* and retained the carboxy-terminal portion of *v-abl*. The results of our experiments showed that the A-MuLV(sd) construct had residual activity in primary lymphoid transformation assays. However, the bone marrow transforming activity of A-MuLV(sd) was at least 25-fold lower than that of A-MuLV, and A-MuLV(sd)-transformed cultures formed continuous cell lines at a very low frequency (4.2%) in comparison to A-MuLV-transformed cultures (71.4%). As only 1 of 13 mice developed A-MuLV(sd)-containing tumors following injection of A-MuLV(sd), this virus was also much less efficient at tumor induction than A-MuLV. These results indicate that the TPK region of *Drosophila abl* is a poor substitute for the TPK region of *v-abl*, even though the amino acid sequences of the two are very similar (87%).

We interpret our results from the A-MuLV(sd) construct to indicate that one of the major determinants of the efficient lymphoid cell-transforming activity of *v-abl* is its TPK region, as was suggested previously by long terminal repeat exchange experiments (15) and by *v-abl/v-src* chimeric viruses (34). To determine whether the viral context of *v-abl* affected disease specificity, Green et al. (15) substituted the U3 sequences of the A-MuLV long terminal repeat with those of the myelo-erythrotropic Friend MuLV or with those of the fibro-erythrotropic Harvey murine sarcoma virus. Analysis of cell surface antigens of tumors induced by these long terminal repeat chimeric viruses showed that the tumors were of pre-B-cell phenotype. These results implied that the pre-B-cell-transforming specificity of A-MuLV was determined by *v-abl*. To identify the sequences responsible for the lymphoid cell-transforming activity of *v-abl*, Mathey-Prevot and Baltimore (34) constructed a *v-abl/v-src* chimeric virus that fused the first 300 amino acids of *v-src* to the TPK and carboxy-terminal regions of *v-abl*. This chimeric virus was active in in vitro lymphoid cell-transformation assays, suggesting that the TPK region of *v-abl* was responsible for its lymphoid cell-transforming activity. However, as the reciprocal construct was transformation defective, it was not possible to conclude definitively that the differences in the biological properties of the *v-abl* and *v-src* TPKs are intrinsic to their TPK regions. The results of our experiments with the A-MuLV(sd) virus allow us to map more precisely the lymphoid cell-transforming activity of *v-abl* to its TPK region. However, neither our results nor the results of Mathey-Prevot and Baltimore (34) exclude the possibility that the efficient lymphoid cell-transforming activity of *v-abl* requires the presence of both the *v-abl* SH2 and TPK domains in *cis*. Indeed, Pawson and colleagues (9, 51) have shown that deletions or substitutions in the SH2 region of the *v-fps* oncoprotein can render it transformation defective, have an inhibitory effect on its kinase activity, and result in restriction of the host range of the Fujinami sarcoma virus. These results indicate that the SH2 region can be an important determinant of the transforming activity of TPK oncogenes.

Our studies with A-MuLV(D) and A-MuLV(sd) also indicate that there are minor determinants of lymphoid cell-transforming activity located in the sequences which make up the carboxy portion of the *v-abl* protein. Possible roles for the carboxy portion of the *v-abl* molecule may include regulation of kinase activity or stabilization of the protein or both. Support for these possibilities comes from our studies of the chimeric *abl* proteins. The results of the in vitro kinase assays indicated that the in vitro kinase activity of the A-MuLV(D) protein showed a marked sensitivity to SDS which was not shared by the *v-abl* or A-MuLV(sd) proteins.

As the only difference between the A-MuLV(D) and A-MuLV(sd) proteins was the presence of *Drosophila abl* carboxy sequences in A-MuLV(D), it is likely that the presence of the *v-abl* carboxy sequences in A-MuLV(sd) acted to modulate the kinase activity or stability of the A-MuLV(sd) protein.

The *Drosophila abl* and *v-abl* TPK regions examined in these studies have only 28 of 209 (13%) nonconserved amino acid residues and contain convenient conserved restriction enzyme sites (Fig. 6). Therefore, it will be possible to use smaller substitutions and in vitro mutagenesis to define more precisely the determinants of lymphoid transformation of *v-abl*. These experiments are in progress.

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