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Insertional Mutagenesis of the Abelson Murine Leukemia Virus Genome: Identification of Mutants with Altered Kinase Activity and Defective Transformation Ability

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A library of Abelson murine leukemia virus (A-MuLV) proviral DNAs with 12- or 6-base-pair (bp) insertional mutations was constructed. The 29 mutations characterized spanned the entire protein-coding region of the provirus. We tested the effects of these mutations both on the kinase activity of the *gag-abl* fusion protein encoded by the provirus and on the ability of the provirus to transform NIH 3T3 fibroblasts. To simplify assessment of the mutant kinases, we expressed the A-MuLV-encoded kinase in the bacterial expression vector pATH2, resulting in production of a *trpE-gag-abl* fusion protein in *Escherichia coli*. We used an immunoprecipitation kinase assay to measure both autophosphorylation and artificial substrate phosphorylation by the mutant kinases. To assay transformation ability of the mutant proviruses, we transfected NIH 3T3 fibroblasts with the mutants and with helper virus (Moloney MuLV) by the DEAE-dextran method. Our analysis of these A-MuLV insertional mutants allows the division of the protein-coding region of the provirus into four domains: domain A (proviral bp 1068 to 1685), in which insertions have no effect on the bacterially expressed kinase, but diminish both kinase activity and transformation efficiency in fibroblasts; domain B (bp 1750 to 2078), in which insertions have no effect on the provirus; domain C (bp 2181 to 2878), the critical kinase domain, in which 12-bp or even 6-bp insertions completely inactivate the A-MuLV kinase and result in transformation-defective proviruses; and domain D (bp 2956 to 4610), the large C-terminal domain in which mutations are silent.

Abelson murine leukemia virus (A-MuLV) is a replication-defective acute transforming retrovirus which causes a rapidly progressive lymphosarcoma in susceptible strains of mice (for reviews, see references 6 and 40). The virus morphologically transforms both pre-B lymphocytes and fibroblastic cell lines in vitro (27, 30). A-MuLV arose by a recombination event between Moloney murine leukemia virus (M-MuLV) and a murine cellular gene, c-*abl*, mapped to chromosome 2 (7, 8, 38). The resultant hybrid genome contains 5′ M-MuLV sequences up to the middle of the p30 domain of *gag*, v-*abl* sequences representing only part of the c-*abl* gene, and M-MuLV sequences again at the 3′ end. This genome codes for a single protein, a *gag-abl* fusion product of M_r ~160,000, whose coding region terminates within the c-*abl*-derived sequences (23). Its only known function is a tyrosine-specific protein kinase activity (32, 41), an enzymatic activity shared with a subset of transforming retroviruses and with receptors for several polypeptide growth factors (11). Cells transformed by A-MuLV have elevated amounts of phosphotyrosine residues in many target proteins (5, 34). However, the substrates relevant for transformation by A-MuLV have yet to be elucidated.

Besides the virus encoding P160, numerous variant strains of A-MuLV have been recovered which encode shorter proteins (10, 25, 28, 29, 42). Previous investigations characterizing these A-MuLV strains and strains deleted by in vitro mutagenesis (21, 34, 39) have revealed an absolute correlation between kinase activity and transformation competence, suggesting that cell transformation by A-MuLV requires activity of the tyrosine kinase. The kinase domain of the provirus has been localized to the 5′ portion of the v-*abl* sequences by homology to other tyrosine kinases (23), by mutagenesis (20), by expression of the kinase in bacteria (4, 37), and by the production of site-specific antibodies which neutralize kinase activity (14). Prior investigations have also suggested a requirement for C-terminal *abl* sequences for efficient lymphoid transformation (21, 28, 29) and for *gag* sequences for protection of the fusion protein from proteolysis in lymphocytes (22).

We constructed and characterized a large number of insertional mutants of A-MuLV to define further the domains of the *gag-abl* fusion protein and their involvement in kinase function and cell transformation. As these mutations might cause less drastic effects than deletion mutations, we were particularly interested to see whether partially active or thermolabile mutant kinases could be obtained. The results enabled a more refined definition of the regions necessary for the function of this viral oncogene to be made.

MATERIALS AND METHODS

Materials. Restriction and other DNA enzymes were obtained from New England BioLabs, Inc. (Beverly, Mass.). Polyclonal rabbit anti-*gag* antiserum (76S 142) was obtained from the Biological Carcinogenesis Branch Repository, National Cancer Institute, Bethesda, Md.

The tyrosine kinase artificial substrate GT(4:1) (2, 24, 44) was obtained from Sigma Chemical Co. (St. Louis, Mo.). Pansorbin was obtained from Calbiochem-Behring (La Jolla, Calif.). Before use in immunoprecipitates, the Pansorbin was

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boiled twice for 30 min in 3% sodium dodecyl sulfate (SDS)–0.1 M NaCl–1.5 M 2-mercaptoethanol–20 mM Tris hydrochloride (pH 7.2) and subsequently washed extensively in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5).

Cell lines. NIH 3T3 fibroblasts, frozen at passage 123, were obtained from the American Type Culture Collection (Rockville, Md.). The fibroblasts were grown at 37°C in 5% CO_2 in Dulbecco modified Eagle medium supplemented with 10% calf serum (GIBCO Laboratories, Grand Island, N.Y.) and 100 μg of gentamicin base per ml. Cells were used for focus assays at our passage 2.

Transfection of NIH 3T3 fibroblasts. Transfections were done by the DEAE-dextran method (19). A-MuLV proviral DNA (1 μg) and M-MuLV proviral DNA (0.5 μg) were suspended in 0.5 ml of phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) plus 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ (PBS plus). A 0.5-ml sample of DEAE-dextran (M_r 500,000; 1 mg/ml in PBS plus; Pharmacia Fine Chemicals, Piscataway, N.J.) was added. NIH 3T3 cells plated the previous day at 5×10^5 cells per 10-cm dish were washed twice with PBS plus and covered with the dextran solution. After 1 h at 37°C, the plates were again gently washed and then the cultures were fed. Cultures were split 1:3 on day 3 after transfection. Confluent cultures were scored for foci of A-MuLV-transformed cells 12 to 16 days after transfection. Controls (M-MuLV proviral DNA alone and wild-type A-MuLV proviral DNA) were performed with each assay.

Cultures enriched for A-MuLV-transformed fibroblasts which contained >50% cells of transformed morphology were obtained by gently washing plates containing foci with medium, which was then transferred to new plates. This method selectively transferred the loosely adherent, rounded-up A-MuLV-transformed cells.

Bacterial hosts and plasmids. *Escherichia coli* HB101 was used as the recipient for most DNA transformations, using standard protocols (17). Strain CC114 (16) was used as the recipient in suppressor-linker insertional mutagenesis.

pT11 contains M-MuLV proviral DNA and flanking cellular DNA cloned into the *Hae*II fragment of pACYC177 (16). pABpv contains full-length (P160) A-MuLV proviral DNA with flanking cellular DNA cloned into the *Eco*RI site of pBR322 (21). pTAB1 was constructed with these two plasmids by excising the A-MuLV genome from pABpv with *Kpn*I (sites in long terminal repeats at proviral base pairs [bp] 479 and 6349) and cloning this fragment into an analogous position in pT11, i.e., after removal of M-MuLV sequences between the *Kpn*I sites.

Plasmid pAAb1 was constructed with the bacterial expression vector pATH2, which was the generous gift of T. J. Koerner and A. Tzagoloff (Columbia University). pTAB1 was digested with *Bst*EII and treated with S1 nuclease to blunt the ends. After the addition of *Bam*HI linkers and digestion with *Bam*HI and *Hin*dIII, the *Bst*EII-*Bam*HI-to-*Hin*dIII fragment of pTAB1 was cloned into the *Bam*HI and *Hin*dIII sites in the polylinker region of pATH2. The resulting plasmid, pAAb1, codes for a fusion protein of *trpE* (~40 kilodaltons) and *gag-abl* (~140 kilodaltons).

DNA enzymatic reactions were performed by standard techniques (18). Large-scale plasmid DNA preparations were performed as previously described (12).

Southern blotting. Southern blots of genomic DNA prepared from enriched cultures of A-MuLV-transformed fibroblasts were performed as previously described (8). The genomic DNA was digested with *Eco*RV (sites at proviral bp 215, 5743, and 6072) with or without *Eco*RI. The probe used, pAb3sub3 (8), was nick translated (26) with four [α-^{32}P]deoxyribonucleoside triphosphates (Amersham Corp., Arlington Heights, Ill.).

Insertional mutagenesis. Plasmids were partially digested with either *Hae*III or *Alu*I, and permuted full-length linears were isolated on 0.5% agarose gels. A 100-fold molar excess of kinased *Eco*RI linkers was ligated to the full-length linears. After heating to 70°C for 5 min, the ligation mix was digested with *Eco*RI and then extracted with phenol-chloroform. The plasmids with linkers attached were separated from free linkers by two precipitations from 2 M ammonium acetate with an equal volume of isopropanol at 24°C. The final pellet was suspended in TE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA), religated, and used to transform HB101 to ampicillin resistance.

Two different *Eco*RI linkers were used: 5'-CCG GAATTCCGG-3' (New England BioLabs) to yield a 12-bp insert, and 5'-GAATTCGAATTC-3' (International Biotechnologies, Inc., New Haven, Conn.) to yield a 6-bp insert. The position of the insert was determined by restriction analysis of crude plasmid preparations (1). To screen for the possibilities of more than one linker insertion or of deletion between two *Hae*III or *Alu*I sites in the initial partial digest, we also utilized restriction analysis to generate small fragments (less than 300 bp) containing the insertion of interest and compared these with the corresponding fragment of the wild-type plasmid.

Libraries of insertional mutations were made in both pTAB1 and pAAb1. Some mutations not recovered in both plasmids were moved from one to the other by transfer of an *Nar*I fragment (proviral bp 1482 to 3260).

Two of the 12-bp insertional mutants used in this study (12-10 and 12-11) were obtained by modification of 16-bp inserts at these sites. These 16-bp inserts were constructed by the suppressor-linker insertional mutagenesis method as previously described (16), but utilizing an aberrant cloned supressor-linker DNA, which yielded a 16-bp insert of 5'-CTGGAATTCCAGCCAG-3' or its complementary sequence. (We are grateful to J. Konopka and L. Hartwell for determining the structure of this aberrant clone.) To convert these to 12-bp insertions, the plasmids were digested with *Eco*RI and treated with S1 nuclease. Blunted full-length linear plasmids were isolated on 0.5% agarose gels. After religation, the plasmids were used to transform HB101 to ampicillin resistance. To confirm the correct conversion, we screened the plasmids obtained both for loss of the *Eco*RI site and for creation of a new *Bal*I site.

Bacterial expression of A-MuLV kinase. Cultures of HB101 containing pAAb1 wild-type or insertional mutant plasmids were grown overnight in M9 medium (33) containing tryptophan (20 μg/ml). A 500-μl sample of this overnight culture was diluted into 4.5 ml of M9 medium lacking tryptophan and incubated at 30°C for 1 h. Indoleacrylic acid (25 μl; 1 mg/ml in ethanol) was added, and the incubation was continued for 2 h.

Extraction of the *trpE-gag-abl* fusion protein was done at 4°C. The bacteria were pelleted by centrifugation, washed in TE buffer plus 0.15 M NaCl, and suspended in 100 μl of TE buffer plus 0.3 M NaCl. Lysozyme (10 μl; 10 mg/ml in TE buffer plus 0.3 M NaCl) was added for a 30-min incubation. An equal volume of 2× Triton lysis buffer (TLB; 10 mM sodium phosphate [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing 2 mM phenylmethylsulfonyl fluoride was added for an additional 30 min. Bacterial debris was removed by centrifugation in a microcentrifuge for 10 min.

The supernatants, containing solubilized fusion protein, were used in the A-MuLV kinase assay.

A-MuLV kinase assay. The same protocol was used for extracting the kinase from either NIH 3T3 fibroblasts 7 days after transfection or enriched cultures of A-MuLV-transformed cells. Approximately 10^6 cells were washed twice with PBS and then solubilized in 0.5 ml of TLB containing 2 mM phenylmethylsulfonyl fluoride for 30 min at 4°C. After clarification by ultracentrifugation (200,000 × *g*, 60 min, 4°C), the supernatants were used in the kinase assay.

A-MuLV kinase activity was measured by a modification of a prior technique (36). Cell or bacterial lysates (as above) were incubated with 100 μl of Pansorbin (20% [vol/vol] in TLB plus 5 mg of bovine serum albumin per ml; Calbiochem) for 1 h at 4°C. After the Pansorbin was pelleted in a microcentrifuge, the supernatants were incubated with polyclonal rabbit anti-*gag* antiserum (1:100 dilution) overnight at 4°C. Pansorbin (100 μl) was then added. After 1 h, the immunoprecipitates were collected by centrifugation in a microcentrifuge. The immunoprecipitates were washed twice with TLB plus bovine serum albumin and then twice with 10 mM Tris hydrochloride (pH 8) and suspended in 50 μl of 50 mM Tris hydrochloride (pH 8).

To assay artificial substrate phosphorylation at the same time as autophosphorylation, we added 25 μl of GT(4:1) (1 mg/ml in 50 mM Tris hydrochloride [pH 8]). GT(4:1) is a synthetic random copolymer of glutamic acid and tyrosine at a molar ratio of 4:1 with M_r 50,000 average (2, 24, 44). The phosphorylation reaction was initiated by adding 25 μl of a reaction mixture to give final concentrations of 20 nM [δ-^{32}P]ATP (5,000 Ci/mmol), 6 mM $MnCl_2$, and 1 mM CTP. After incubation at 24°C for 10 to 30 min, the reaction was terminated by adding 10 μl of stopping solution to give final concentrations of 10 mM ATP, 10 mM EDTA, and 20 mM Tris hydrochloride (pH 8). The immunoprecipitates were pelleted in a microcentrifuge. Samples (75 μl) of the supernatant containing phosphorylated GT(4:1) were spotted onto squares of Whatman 3 mm filter paper which were placed in 10% trichloroacetic acid containing 10 mM sodium PP_i. After extensive washing in this solution, the papers were rinsed in ethanol and dried, and ^{32}P incorporation into GT(4:1) was determined by scintillation counting.

After removal of the remaining supernatant, the immunoprecipitates were washed once in TLB containing 5 mg of bovine serum albumin per ml and 0.1% SDS and then suspended in 100 μl of electrophoresis sample buffer (36) and heated to 100°C for 10 min. After electrophoresis on 6% polyacrylamide-SDS gels (15), autophosphorylation was visualized by autoradiography.

Metabolic labeling of A-MuLV proteins. NIH 3T3 fibroblasts were transfected with A-MuLV and M-MuLV proviral DNAs as described above. Twelve days after transfection, the fibroblasts (10^6/10-cm plate) were incubated in methionine-free medium for 20 min and then with medium containing [^{35}S]methionine (150 μCi/3 ml) for 1 h. The fibroblasts were lysed in ice-cold TLB supplemented with 0.5% sodium deoxycholate, 0.1% SDS, and 2 mM phenylmethylsulfonyl fluoride. The remainder of the preparation was done at 4°C. After clarification by ultracentrifugation (200,000 × *g*; 3 h), the extracts were incubated with 10 μl of normal goat serum (2 h) and then with 50 μl of Pansorbin (1 h). The immunoprecipitates were pelleted in a microcentrifuge, and the supernatant was incubated overnight with 5 μl of anti-Rauscher murine leukemia virus p15 antiserum (National Institutes of Health serum repository no. 78S 249). After incubation with 25 μl of Pansorbin, the immunoprecipitates were pelleted, washed twice in lysis buffer, suspended in electrophoresis sample buffer (36), and heated to 97°C for 10 min. The samples were electrophoresed on 6% resolving gels. Before drying, the gels were soaked in 1 M sodium salicylate for 1 h. Labeled proteins were visualized by autoradiography.

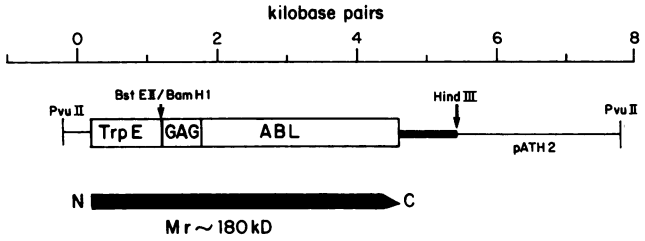

FIG. 1. Bacterial expression plasmid encoding *gag-abl*. The bacterial expression plasmid pAAb1 was constructed by cloning the *Bst*EII-to-*Hin*dIII fragment of pTAB1 (containing the majority of *gag-abl* encoding sequences) into the polylinker region of pATH2, as described in Materials and Methods. This plasmid encodes a fusion protein of *trpE-gag-abl* of predicted M_r ~180,000. kD, Kilodaltons.

Heat inactivation of A-MuLV kinase. To test for thermolabile kinase activity, we heated washed immunoprecipitates to 40°C for 0 to 10 min before the kinase assay.

RESULTS

Construction of plasmids containing A-MuLV oncogene. We constructed plasmid pTAB1, which contains a full-length copy of A-MuLV proviral DNA (P160 strain) in the small vector pACYC177 (see Materials and Methods), for insertional mutagenesis. pTAB1 contains no *Eco*RI restriction sites, allowing the insertion of *Eco*RI linkers and convenient mapping of their position.

To simplify the assay of tyrosine kinase activity encoded by our insertional mutants, we constructed a bacterial expression plasmid. The *Bst*EII-to-*Hin*dIII fragment of pTAB1 containing the majority of the protein-coding region of A-MuLV was cloned into the polylinker region of the expression vector pATH2 (T. J. Koerner, unpublished data). The resulting plasmid, pAAb1 (Fig. 1), codes for a fusion protein of *trpE* and *gag-abl* of predicted M_r ~180,000. Compared with the protein-coding sequence of v-*abl*, the *gag-abl* of this plasmid lacks only the first 109 bp of *gag* sequence. This plasmid also contains minimal vector sequences and no *Eco*RI sites, facilitating insertional mutagenesis by our methods.

Bacterial expression of A-MuLV tyrosine kinase. To test for autophosphorylation of the bacterially expressed A-MuLV kinase, bacteria containing pATH2 or pAAb1 were lysed in Triton X-100 and the soluble extract was immunoprecipitated with antiserum to *gag* and incubated with [δ-^{32}P]ATP and Mn^{2+}. The resultant phosphoproteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The extract from bacteria containing pATH2 demonstrated essentially no detectable phosphoproteins (Fig. 2, lane 1). The extract from bacteria containing pAAb1 (Fig. 2, lane 2) revealed three major phosphoproteins at M_rs 180,000, 135,000, and 120,000. The M_r-180,000 phosphoprotein corresponds to the predicted full-length *trpE-gag-abl* fusion protein. These three phosphoproteins were also immunoprecipitated with antiserum to *trpE* (data not shown), suggesting that the lower-molecular-weight bands represent degrada-

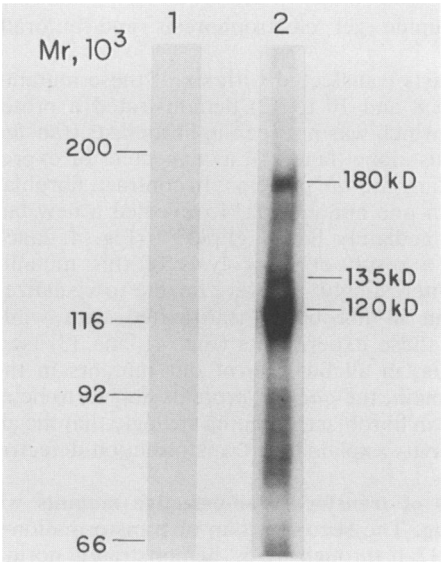

FIG. 2. Autophosphorylation of the *trpE-gag-abl* fusion protein expressed in bacteria. Triton X-100 extracts of HB101 containing either pATH2 (lane 1) or pAAb1 (lane 2) were immunoprecipitated with antiserum to *gag* and assayed for autophosphorylation as described in Materials and Methods. The resulting phosphoproteins were analysed by SDS-polyacrylamide gel electrophoresis. Shown is an autoradiogram of the gel. kD, Kilodaltons.

tion products of the 180-kilodalton fusion protein, with removal of C-terminal portions of the kinase. Such instability of the C-terminal portion of the A-MuLV kinase in bacteria has been previously observed (37).

We also tested the ability of the bacterially produced A-MuLV kinase to phosphorylate the exogenous substrate GT(4:1), a random copolymer of glutamic acid and tyrosine at a 4:1 molar ratio with M_r 50,000 average (2, 24, 44). GT(4:1) was accepted as a substrate by the kinase, with K_m = 50 μM. In a representative assay with extracts from bacteria containing pATH2, 1,260 cpm of ^{32}P were incorporated into GT(4:1). Extracts from bacteria containing pAAb1 yielded 16,890 cpm incorporated into GT(4:1).

Construction of insertional mutants. Libraries of 12-bp insertions in both pTAB1 and pAAb1 were constructed by ligating *Eco*RI linkers onto permuted full-length linear plasmids obtained by partial digestion with frequent-cutting enzymes (*Hae*III, *Alu*I). After digestion of the added linkers with *Eco*RI, the linear plasmids were closed with DNA ligase and used to transform HB101 to ampicillin resistance. The insertion rate was quite efficient, with >80% of screened colonies containing new *Eco*RI sites. Of 228 colonies containing inserts which were screened, 136 contained inserts in the protein-coding sequences of these plasmids. The 12-bp insertions were mapped by restriction analysis and were found to span the entire protein-coding region of the provirus (Fig. 3).

Restriction analysis to determine the length of the insert revealed that less than 5% of the screened clones contained either two tandem linker insertions (i.e., 24 bp) or a deletion between two adjacent *Hae*III or *Alu*I sites.

For further analysis of the kinase domain of A-MuLV, a library of 6-bp inserts in pAAb1 was constructed in a similar manner. Six of these insertional mutants mapped to the kinase domain (Fig. 3, legend) and were characterized further.

Kinase activity of insertional mutants. The 12-bp insertional mutants of pAAb1 (Fig. 3) were tested for kinase activity by the bacterial expression kinase assay. Mutations 12-2 through 12-10, spanning *gag* sequences and N-terminal *abl* sequences through proviral bp 2078, showed fully active autophosphorylation and artificial substrate phosphorylation by the expressed kinase (Table 1). Similarly, insertions located in the C-terminal portion of the A-MuLV kinase from bp 2956 to the C terminus of the protein failed to affect tyrosine kinase activity.

In contrast, mutants 12-11 (bp 2181) through 12-17 (bp 2878) were entirely inactive in the kinase assay (Table 1). To ensure that we were not overlooking mutants with an increased K_m for ATP which might be inactive under our assay condition of 10 nM ATP, we also tested these mutants at 5 μM ATP. Again, none demonstrated any detectable autophosphorylation or GT(4:1) phosphorylation, whereas the activity of the wild-type enzyme was easily detectable (data not shown). Thus, these mutations define a critical kinase domain of A-MuLV from proviral bp 2181 to bp 2878 which is intolerant of 12-bp insertions.

To analyze this critical kinase domain further, we constructed five mutants with 6-bp insertions in this domain (Fig. 3, legend). However, like the 12-bp insertions in this region, none of these 6-bp insertional mutants showed any detectable autophosphorylation or GT(4:1) phosphorylation as assayed by bacterial expression, even at 5 μM ATP (data not shown). Thus, the critical kinase domain is extremely sensitive and intolerant of even slight alterations.

Transformation ability of A-MuLV insertional mutants. The 12-bp insertional mutants of pTAB1 were tested for their ability to transform NIH 3T3 fibroblasts. A-MuLV mutants were mixed with M-MuLV DNA as helper and introduced into cells by the DEAE-dextran method (19). This permits efficient viral spread of A-MuLV through the culture and yields improved efficiency of A-MuLV focus formation compared with the use of calcium phosphate coprecipitation with a selectable marker (9). Confluent cultures of fibroblasts were scored for foci 12 to 16 days after transfection.

Mutants 12-6 through 12-10, with inserts in the p30 portion of *gag* and those *abl* sequences on the N-terminal side of the critical kinase domain, were fully active in focus formation (Table 1). Similarly, mutants 12-18 through 12-29, with inserts C terminal to the critical kinase domain, were also fully active. Mutants 12-11 through 12-17, which were devoid of kinase activity in the bacterial extracts, were totally inactive in focus formation.

Insertional mutations in p15 and p12 portions of *gag* (mutants 12-1 through 12-5) caused a more than 10-fold

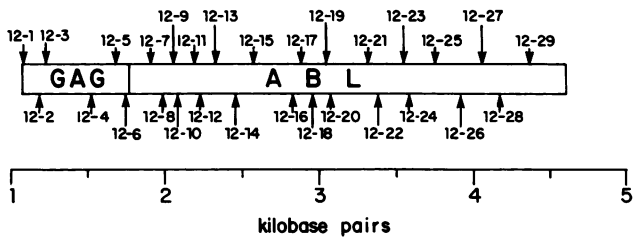

FIG. 3. Library of 12-bp insertional mutants of A-MuLV. The positions of 12-bp insertional mutations in plasmids pTAB1 and pAAb1 are indicated by arrows for mutants 12-1 through 12-29. Five 6-bp insertional mutants were also characterized; these insertions were at sites corresponding to 12-12, 12-14, 12-16, the *Hae*III site at proviral bp 2663, and the *Alu*I site at bp 2779.

TABLE 1. Analysis of 12-bp insertional mutants of A-MuLV[a]

Mutation[b]		Kinase activity[c]		Focus-forming activity[d] (foci/plate)
No.	Location (bp)	Auto	GT (4:1)	
12-1	1071 H	NT[e]	NT	1–5
12-2	1195 H	+	+	1–5
12-3	1216 H	+	+	1–5
12-4	1522 H	+	+	1–5
12-5	1685 H	+	+	1–5
12-6	1750 H	+	+	>100
12-7	1905 H	+	+	>100
12-8	1978 A	+	+	>100
12-9	2052 H	+	+	>100
12-10	2078 A	+	+	>100
12-11	2181 H	−	−	0
12-12	2234 H	−	−	0
12-13	2329 A	−	−	0
12-14[f]	2459 H	−	−	0
12-15	2577 H	−	−	0
12-16	2839 A	−	−	0
12-17	2878 H	−	−	0
12-18	2956 A	+	+	>100
12-19	3044 A	+	+	>100
12-20	3076 A	+	+	>100
12-21	3306 H	+	+	>100
12-22	3369 A	+	+	>100
12-23	3549 A	+	+	>100
12-24	3581 H	+	+	>100
12-25	3752 H	+	+	>100
12-26	3909 H	+	+	>100
12-27	4053 A	+	+	>100
12-28	4169 H	+	+	>100
12-29	4366 A	+	+	>100

[a] 12-bp insertional mutants 12-1 through 12-29 were assayed for kinase activity (bacterial expression) and focus-forming activity as described in Materials and Methods.

[b] Locations of the mutations are given at the proviral base pair at which the restriction site begins; H and A represent mutations at *Hae*III and *Alu*I sites, respectively.

[c] Mutants were scored as positive for autophosphorylation in the bacterial expression kinase assay when demonstrating autophosphorylated bands of comparable intensity to that of the simultaneous wild-type control. The negative mutants all showed undetectable autophosphorylation. Mutants were scored as positive for GT(4:1) phosphorylation if the level of ^{32}P incorporation (counts per minute) into the substrate was >50% that of the simultaneous wild-type control. The negative mutants all showed undetectable incorporation.

[d] In the focus assay, wild-type A-MuLV yielded >100 distinct foci per 10-cm plate 12 to 16 days after transfection; controls performed with helper virus alone did not yield foci.

[e] NT, Not tested. Mutant 12-1 was not tested for kinase activity as this site was not present in the bacterial expression plasmid used. This mutant was tested for kinase activity present in extracts of transfected fibroblasts (Fig. 5 and 6).

[f] Mutant 12-14 encodes a truncated *gag-abl* protein (Fig. 4).

decrease in focus-forming activity compared with that of wild-type A-MuLV (Table 1). The foci formed were small and delayed in appearance (2 to 3 days) compared with wild-type foci.

Analysis of transformation-defective mutants with insertions in the critical kinase domain. One group of transformation-defective mutants, 12-11 through 12-17, was defective in kinase activity as well, suggesting that an inactive kinase was responsible for the transformation-defective phenotype. An alternative explanation, however, was that the *gag-abl* proteins encoded by these mutants might be poorly expressed or unstable. To address this possibility, we labeled fibroblasts transfected with these mutants with [^{35}S]methionine, and the encoded kinases were extracted, immunoprecipitated with antiserum to *gag*, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 4).

Fibroblasts transfected with six of these mutants (Fig. 4, lanes 6 to 8 and 10 to 12) demonstrated a protein of M_r ~150,000 which was not seen in fibroblasts transfected with helper virus alone (lane 14), as expected for expressed and stable mutant *gag-abl* proteins. In contrast, fibroblasts transfected with one mutant, 12-14, revealed a new band at M_r 75,000 immediately below gPr80^{gag} (Fig. 4, lane 9). This might be a result of proteolysis of this mutant *gag-abl* protein. Interestingly, we were unable to visualize the *gag-abl* protein in fibroblasts transfected with wild-type A-MuLV in these experiments (Fig. 4, lane 13) (see Discussion). Thus, in all but one of our mutants in the critical kinase domain, the *gag-abl* proteins appear to be expressed and stable in fibroblasts, arguing strongly that the absence of kinase activity explains the transformation-defective phenotype.

Analysis of transformation-defective mutants with insertions in gag. The second group of transformation-defective mutants, 12-1 through 12-5, demonstrated normal kinase activity in the bacterial expression assay. To assess the kinase activity encoded by these mutants in fibroblasts, we prepared extracts of NIH 3T3 fibroblasts 7 days after transfection and performed an immunoprecipitation kinase assay. The resulting phosphoproteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 5).

Cells transfected with wild-type A-MuLV and helper virus (Fig. 5, lane 2) contained a phosphoprotein of M_r ~150,000 which was not present in cells transfected with helper virus alone (lane 1); this represents the autophosphorylated A-MuLV kinase. Extracts from fibroblasts transfected with *gag* insertional mutants 12-1 through 12-5 (lanes 3 to 7, respectively) revealed no detectable kinase autophosphorylation. In contrast, extracts from fibroblasts transfected with mutants having normal kinase activity and transformation ability (lanes 8 and 9) had easily detectable autophosphorylation.

Fibroblasts transfected with the *gag* insertional mutants were also analyzed by [^{35}S]methionine labeling (Fig. 4). Unlike the mutants with insertions in the critical kinase domain in which the *gag-abl* protein was readily visualized (Fig. 4, lanes 6 to 12), the *gag* insertional mutants demonstrated no detectable protein (Fig. 4, lanes 1 to 5).

To summarize the phenotype of the transformation-defective mutants with insertions in *gag*, the encoded kinases are expressed and active in bacteria. However, no active kinases can be extracted from fibroblasts transfected with these mutants, and the *gag-abl* proteins cannot be visualized in these fibroblasts by [^{35}S]methionine labeling. The most likely explanation for these findings is that insertions in *gag* render the *gag-abl* protein unstable or poorly expressed in fibroblasts. These data are reminiscent of the previously noted requirement for *gag* sequences to protect the *gag-abl* protein against proteolysis, as described in lymphocytes (22).

Despite the low efficiency of fibroblast transformation by these *gag* insertional mutants, we were able to recover enriched lines of cells transformed by four of these mutants. Southern blot analysis of genomic DNA from one of these lines (containing mutant 12-5) revealed the continued presence of the *Eco*RI site (i.e., inserted linker) in the integrated proviral DNA (data not shown). Thus, the small number of foci caused by this mutant are not due to reversion events associated with excision of the 12-bp insert.

In contrast to the experiments described above, in which

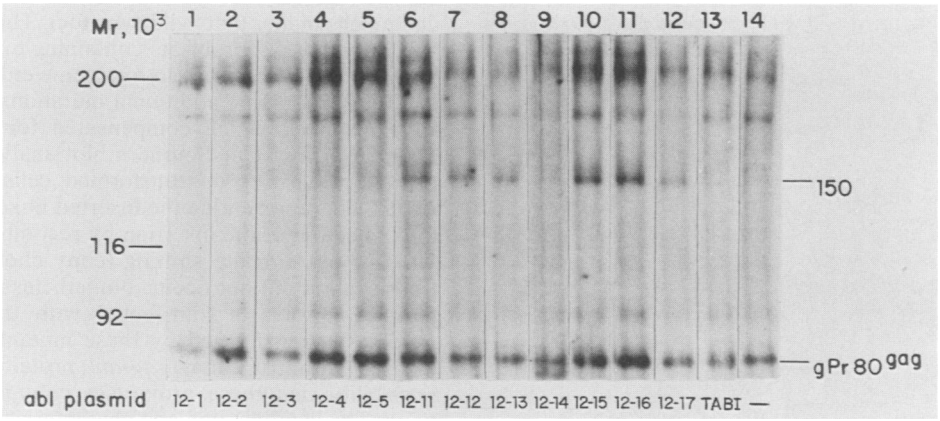

FIG. 4. [^{35}S]methionine labeling of transformation-defective mutants. Fibroblasts transfected with the indicated *abl* plasmids and M-MuLV proviral DNA as helper were labeled with [^{35}S]methionine. Extracts of these fibroblasts were immunoprecipitated with antiserum to *gag* and electrophoresed on 6% resolving gels. The labeled proteins were visualized by autoradiography as shown.

no active kinase could be extracted from the bulk population of fibroblasts transfected with the *gag* insertional mutants, we were able to extract active kinases from the rare fibroblasts transformed by these mutants. Figure 6 shows an autoradiogram from an immunoprecipitated kinase assay performed on extracts from fibroblasts transformed by four of these mutants. 12-1 (lane 1) and 12-3 (lane 2) demonstrate an autophosphorylated kinase of M_r 150,000 identical to that encoded by wild-type kinase (lane 5); 12-4 (lane 3) and 12-5 (lane 4) encode kinases of M_rs 120,000 and 100,000. In addition, all the lanes contain phosphoproteins of lower M_r compatible with either degradation of the kinase or proviral mutations resulting in truncated *gag-abl* proteins. One possibility for the *gag* insertional mutants is that some alterations in the viral DNA or in its expression are required to yield a transformed phenotype. The nature of these events is under further study.

DISCUSSION

We constructed and characterized a large number of insertional mutants of A-MuLV. The results may be summarized by a division of the *gag-abl* fusion protein into four domains (Fig. 7): domain A, containing the majority of *gag* sequences (p15 and p12, proviral bp 1068 to 1685); domain B, with the remaining *gag* sequences (p30) and 5′ *abl* sequences (bp 1750 to 2078); domain C, representing the critical tyrosine kinase domain (bp 2181 to 2878); and a large domain D, containing *abl* sequences C terminal to the kinase domain (bp 2956 to 4610). Zeigler et al. (43) have previously designated five regions (I to V) of A-MuLV proviral DNA on largely structural criteria. Since our designation of domains was based on functional criteria we could not incorporate them into the previously defined domains and have thus designated new ones (A to D).

Domain A. The five mutants with 12-bp insertions in domain A reveal a previously unrecognized requirement for A-MuLV *gag* sequences for efficient fibroblast transformation. We were surprised with the partially transformation-defective phenotype of these mutants since the bacterially encoded kinases demonstrated normal activity. However, we were unable to extract active kinases from fibroblasts transfected with these mutants. This suggests that the *gag-abl* proteins expressed by these mutants in fibroblasts either had greatly diminished kinase activity (unlike the bacterially expressed enzyme) or were poorly expressed or unstable.

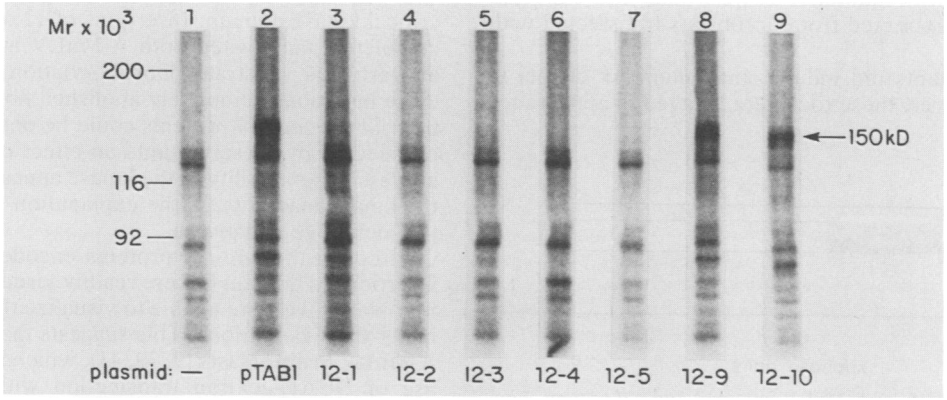

FIG. 5. Autophosphorylation of the A-MuLV kinase extracted from transfected fibroblasts. Fibroblasts transfected with the indicated plasmids and M-MuLV proviral DNA as helper were extracted and assayed for autophosphorylation of the A-MuLV kinase as described in Materials and Methods. Shown is an autoradiogram of the gel. kD, Kilodaltons.

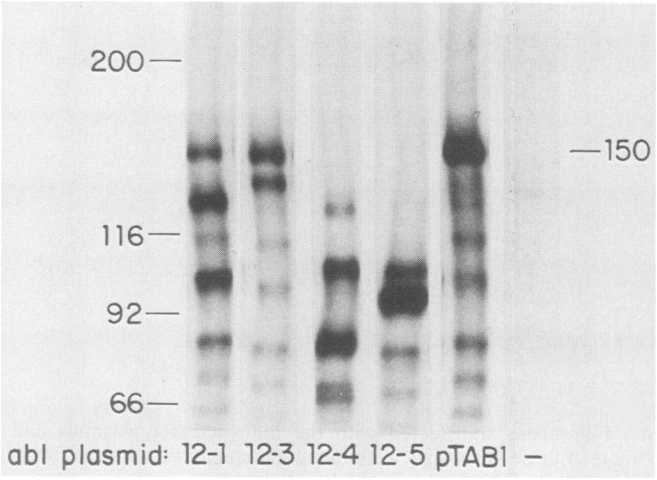

FIG. 6. Autophosphorylation of the A-MuLV kinase extracted from fibroblasts transformed by the *gag* insertional mutants. Fibroblasts transformed by *gag* insertional mutants (lanes 1 to 4), by wild-type A-MuLV (lane 5), or containing helper virus alone (lane 6) were extracted and assayed for kinase autophosphorylation as described in Materials and Methods. An autoradiogram is shown.

To distinguish between these two possibilities, we performed metabolic labeling of the *gag-abl* protein in fibroblasts 12 days after DEAE-dextran transfection with A-MuLV and M-MuLV proviral DNA. Unlike the other transformation-defective, kinase-negative mutants (those in domain C), the mutants in domain A yielded no demonstrable *gag-abl* protein in fibroblasts. Thus, rather than postulating that the kinases expressed in bacteria and fibroblasts differ in activity, it appears more likely that these mutant kinases are poorly expressed or unstable in fibroblasts. We suspect that these mutations result in increased susceptibility of the *gag-abl* fusion protein to proteolysis, perhaps by interfering with the normal folding of the *gag* domain. Such an effect has previously been observed with 12-bp insertions in *gag* studied in lymphocytes (22).

Mutant 12-1 alone has a possible second explanation for defective transformation, as the insertion is immediately after the glycine acceptor for myristic acid (31). It is possible that the insertion interferes with proper myristylation of the *gag-abl* protein and therefore prevents its proper targeting to the inner face of the plasma membrane. However, if this was the sole problem with mutant 12-1, we would have expected an active kinase extracted from fibroblasts transfected with this mutant.

These *gag* mutants did induce small numbers of foci in fibroblasts; however, these foci were delayed in appearance and much smaller than wild-type foci. This might represent a small number of fibroblasts containing high enough levels of expression of the kinase to overcome enhanced proteolysis. Alternatively, some additional mutations in the virus might have occurred which compensated for the effects of the 12-bp insertion. Our Southern blot analysis of one of these mutant proviruses in transformed cells revealed that the *Eco*RI site representing the inserted linker was still present; thus, deletion of the insertion by recombination with helper virus or by template shifting (copy choice) during reverse transcription did not occur. Nonetheless, in contrast to the bulk of fibroblasts transfected with these mutants, rare fibroblasts transformed by these mutants contained active kinases with truncated *gag-abl* proteins. This raises the possibility that these proviruses contain secondary mutations. The identification and characterization of these potential additional mutations will require the cloning and sequencing of proviral DNA from the transformed cell lines.

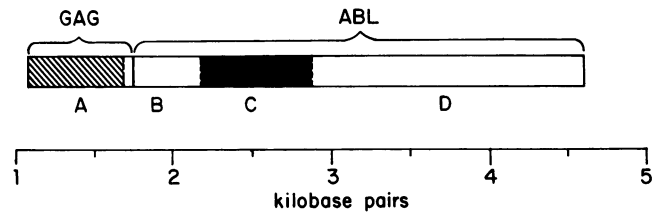

FIG. 7. Domains of the A-MuLV-encoded *gag-abl* protein. Four functional domains of the A-MuLV-encoded P160 protein are indicated: A (proviral bp 1068 to 1685), B (bp 1750 to 2078), C (bp 2181 to 2878), and D (bp 2956 to 4610). See Discussion for details.

Finally, previous studies of A-MuLV deletion mutants suggested that *gag* sequences were not essential for fibroblast transformation; a mutant with a deletion of all but the first 34 amino acids of *gag* sequence still transformed NIH 3T3 fibroblasts normally (21). Our insertional mutants suggest that some regions of *gag*, when altered by mutation, can prevent the normal function of the *gag-abl* protein. Furthermore, our data emphasize the point that deletions and insertions as mutations may yield distinct information.

Domain B. Domain B contains the remaining *gag* sequences (p30) as well as the initial 300 bp of c-*abl* sequence. Insertions in this region had no effect on kinase function or fibroblast transformation. Thus, this area of A-MuLV has no clear function. Interestingly, the homology of c-*abl* to other tyrosine kinases, although most extensive in the critical kinase domain, begins within the c-*abl* sequences in domain B, immediately after the *gag-abl* junction (23). This suggests that these sequences have some role in tyrosine kinase function. However, our analysis with 12-bp insertions did not detect any such role.

Domain C. Domain C (proviral bp 2181 to 2878) represents the critical area of A-MuLV for kinase function; this domain contains the ATP-binding site of the kinase (bp 2175 to 2192) as well as a major site of tyrosine autophosphorylation (bp 2510 to 2512). This area is similar to, although slightly smaller than, the minimal kinase domain defined in previous studies by either bacterial expression of the kinase (37) or deletion-insertion analysis (20). The sequence homology of c-*abl* to other tyrosine kinases is extensive in domain C and ends at proviral bp 2888 (23), close to our defined end of the critical kinase domain. Insertions of 12 or 6 bp in domain C completely inactivated both A-MuLV autophosphorylation and artificial substrate phosphorylation, and in each case these insertions completely abolished fibroblast transformation. Since *gag-abl* proteins could be detected in fibroblasts transfected by these mutants an effect of the mutations on expression or stability of the kinase appears unlikely, leaving the kinase inactivity as the explanation for the transformation-defective phenotype.

Although the *gag-abl* proteins encoded by mutants with insertions in domain C were readily visualized in transfected fibroblasts, we were unable to visualize the wild-type protein under these conditions. This suggests that the toxic effect of A-MuLV in fibroblasts (9, 39, 43), which is diminished by the use of DEAE-dextran transfection with helper virus (9), might not be totally abolished.

The exquisite sensitivity of domain C to insertional inactivation suggests an extensive secondary and tertiary struc-

ture necessary for kinase function. It is of interest that no disassociation of autophosphorylation and transphosphorylation was observed in this study; both were abolished with mutations in the critical kinase domain, and neither were affected by mutations in domains B and D. This suggests that the substrate-binding site of the protein is also encoded within the critical kinase domain. The absolute concordance of kinase function and transformation competence reaffirms the role of the kinase in mediating cell transformation by v-*abl*.

Finally, one of our goals in this study was to obtain mutants with partially inactive or thermolabile kinases; similar insertional mutagenesis of M-MuLV has yielded mutants temperature sensitive for plaque formation (3; N. Tanese, personal communication). We were somewhat surprised to find that the mutations clearly segregated into two groups, either fully active or fully inactive. We tested the active kinases with inserts surrounding the critical kinase domain for temperature sensitivity; none were thermolabile (data not shown). The critical kinase domain per se was intolerant of even 6-bp insertions. The other domains appeared to have no influence on kinase function, suggesting totally independent folding of the two domains surrounding the kinase domain. Recently, two other groups have been successful in obtaining temperature-sensitive tyrosine kinase mutants of A-MuLV using insertional mutagenesis (13) or chemical mutagenesis (35).

Domain D. Domain D of A-MuLV contains sequences 3′ to the critical kinase domain and is the largest domain. As in previous studies characterizing viral strains with 3′ deletions (10, 21, 39), this area appeared totally unnecessary for either kinase function or fibroblast transformation. This domain has previously been found to be involved in lymphocyte transformation, as deletions of it greatly reduced the efficiency of lymphocyte transformation (21, 28, 29). In addition, this domain has also been proven responsible for the toxic effect of A-MuLV in NIH 3T3 fibroblasts (9, 39, 43). Further characterization of our insertional mutants in domain D may aid in specifically defining which C-terminal sequences are important in particular lymphoid cell types and which C-terminal sequences result in toxicity to fibroblasts.

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