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Site-Directed Deletions of Abelson Murine Leukemia Virus Define 3′ Sequences Essential for Transformation and Lethality

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Abelson murine leukemia virus (A-MuLV) encodes a single protein with tyrosine kinase activity that can transform fibroblast cell lines in vitro and lymphoid target cells in vitro and in vivo. Expression of kinase-active A-MuLV protein can result in a deleterious effect on transformed fibroblast populations, leading to cell death or selection for nonlethal mutants of the virus. These mutants retain expression of the kinase activity but have lost large portions of the carboxy terminus of the Abelson protein. To more precisely map the sequences involved in this lethal effect, we have isolated a series of site-directed deletions from a DNA clone of the P160 wild-type strain of A-MuLV. In addition, a number of unexpected, spontaneous deletions occurring during transfection of NIH 3T3 cells were isolated. These deletions result in expression of carboxy-terminal truncated forms of the A-MuLV protein ranging from 130,000 to 84,000 in molecular weight. Analysis of the transforming and lethal activities of each mutant recovered in its RNA viral form shows that the transformation-essential and lethal-essential sequences do not overlap. These data and our previous work suggest that a function carried by the carboxy-terminal region of the A-MuLV protein acts in *cis* with the kinase-essential region to mediate the lethal effect.

Abelson murine leukemia virus (A-MuLV) is a replication-defective, highly oncogenic retrovirus capable of rapid in vivo and in vitro transformation of bone marrow lymphoid cells (1, 17). A-MuLV arose through a recombination event between the genome of a replication-competent retrovirus (Moloney murine leukemia virus [M-MuLV]) and a host cell gene (c-*abl*) (4, 7). The A-MuLV genome encodes a single polypeptide. The molecular weight of the polypeptide varies between different mutant and wild-type strains of A-MuLV. Two wild-type strains of A-MuLV exist; one produces a polypeptide of 120,000 daltons (P120) and the other produces a polypeptide of 160,000 daltons (P160). Approximately 30 kilodaltons at the amino terminus of the polypeptide are of M-MuLV *gag* gene origin (16, 27), whereas the remaining 90 to 130 kilodaltons are derived from the c-*abl* sequences (9, 18).

The polypeptide is phosphorylated in vivo (15, 27) and has an autokinase activity in vitro (24). The level of in vitro autokinase activity roughly correlates with lymphoid target cell-transforming efficiency in vitro (18). Mutant strains of P120, such as P90 and P100 which have suffered carboxy-terminal truncations, have lower in vitro kinase levels and lower transforming efficiencies than do wild-type strains. Since these truncation mutants are still capable of transforming, albeit at a lower efficiency, the carboxy-terminal A-MuLV sequences do not appear to be essential for transformation.

The carboxy-terminal sequences do appear, however, to be important for modulating some cellular responses to A-MuLV transformation. Infection of some rodent fibroblastic cell lines (notably one subline of BALB/c 3T3) by wild-type A-MuLV (P160) has a deleterious effect which results in cell death for a significant fraction of infected cells. As a consequence, infection of this cell line results in a strong selection for viral mutants encoding truncated viral proteins (29). It has been observed that infection of this cell line with wild-type A-MuLV (P160) initially results in transformation but that, under nonselective conditions, the transformed phenotype is lost after 3 to 4 weeks, apparently due to the slower growth or death of transformed cells and overgrowth of the culture by cells which have lost the integrated A-MuLV provirus. This deleterious or lethal effect is also seen in NIH 3T3 cells when direct transformation with cloned DNA of A-MuLV is carried out (8). When the A-MuLV-transformed cells are maintained under growth conditions which

strongly select for the transformed phenotype, surviving cells often contain not the original wild-type A-MuLV but a class of mutants with large carboxy-terminal truncations of about 60 kilodaltons. This new class of mutants produces a level of in vitro autokinase activity similar to that of the wild type. The relative efficiencies of lymphoid and fibroblast transformation are also approximately that of wild-type A-MuLV (29).

The carboxy-terminal deletion mutants, therefore, appear to differ from previously isolated A-MuLV mutants. Their large deletions help to bracket an area whose removal does not appear to grossly affect transformation but which is important in controlling the deleterious effect of A-MuLV infection. This deleterious effect constitutes an alternative phenotypic response to A-MuLV infection and is potentially useful for understanding the cellular response to the A-MuLV-transforming protein. For this reason, we are interested in developing a series of A-MuLV mutants which can be used to study the response of target cells to transforming proteins altered in this region. Our approach has been to create truncation mutants of various sizes in vitro on cloned A-MuLV DNA and to recover the mutant viruses through transfection. This approach has allowed recovery of A-MuLV mutants not found by standard selection procedures. Biological, protein, and DNA structure analysis of these mutants has defined the borders of the sequences controlling the lethal effect and the 3′ sequences essential for kinase activity and transformation.

MATERIALS AND METHODS

Cells and viruses. The BALB-1 line was subcloned from the BALB/c 3T3 line from the American Type Culture Collection. The NIH 3T3 cell line and A-MuLV strains P160, P120, P106, and P92td were as previously described (19, 25, 29).

Plasmids and bacteria. Plasmids pAB3sub3 (7) and pZAP and phage Ch21AP160 (a single long-terminal-repeat proviral circle cloned via the unique *Hin*dIII site) were generous gifts of S. Latt, S. Goff, D. Baltimore, J. Hoffman, and R. Weinberg. The cloned P120 genome was generously contributed by S. Aaronson (22).

The unique *Sal*I site was removed from pBR322 by treating *Sal*I-digested plasmid with *Bal*31 (Bethesda Research Laboratories, Inc.), which functions both as a single-stranded endonuclease and a double-stranded exonuclease (12). Religation was carried out by T4 DNA ligase under dilute DNA concentrations (0.3 μg/ml) to enhance unimolecular ligations. The ligated DNA was then redigested with *Sal*I to lower the transforming efficiency of plasmids not deleted at the *Sal*I site and transformed into $CaCl_2$-shocked (13) *Escherichia coli* HB101. The resulting colonies were screened for the ampicillin-resistant, tetracycline-sensitive phenotype. A plasmid lacking the *Sal*I site (pBR322Δ*Sal*I) was selected and purified by cesium chloride-ethidium bromide centrifugation.

Insertion of the A-MuLV P160 genome into pBR322Δ*Sal*I was carried out by digestion of Ch21AP160 and pBR322Δ*Sal*I with *Hin*dIII (Bethesda Research Laboratories, Inc.) (20 mM Tris [pH 7.4], 7 mM $MgCl_2$, 60 mM NaCl) followed by calf intestinal phosphatase (Boehringer Mannheim) treatment of pBR322Δ*Sal*I. Ligation reactions (0.25 μg of pBR322Δ*Sal*I per 0.02 ml, 2 μg of Ch21AP160 per 0.02 ml, 50 mM Tris [pH 7.5], 10 mM dithiothreitol, 5 mM $MgCl_2$, 0.8 mM ATP) were incubated at 15°C. DNA from the ligation reaction was transformed into $CaCl_2$-shocked *E. coli* HB101. Colonies were screened by the minilysate technique, and a plasmid containing the A-MuLV genome, called pAB160(Δ*Sal*I) was selected and purified by cesium chloride-ethidium bromide equilibrium centrifugation.

Deletion mutagenesis of the wild-type P160 genome. Approximately 15 μg of pAB160(Δ*Sal*I), pBR322Δ*Sal*I with the P160 genome as insert, was digested with 40 U of *Sal*I (Bethesda Research Laboratories, Inc.) for 60 min at 37°C. The digested DNA was extracted twice with Tris-buffered phenol, pH 8, and twice with $CHCl_3$-isoamyl alcohol (24:1, vol/vol). The DNA was then ethanol precipitated, centrifuged, and dried under vacuum. *Bal*31 buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 12 mM $CaCl_2$, 1 mM EDTA) was used to resolubilize the DNA to a final concentration of 0.075 μg/μl. This reaction mix was preincubated at 30°C for 10 min. *Bal*31 was added (0.2 U/μg of DNA), and aliquots were removed at 1, 2, and 5 min. Reactions were stopped by pipetting directly into Tris-buffered phenol. DNA from each aliquot was phenol extracted twice, $CHCl_3$-isoamyl alcohol extracted twice, ethanol precipitated, centrifuged, and dried under vacuum. The aliquots were resolubilized together in 10 mM Tris, pH 8.0, and an aliquot was removed for *Xba*I digestion. *Xba*I-digested fragments were run on 0.8% agarose gels and examined for changes in mobility due to *Bal*31 treatment. The rest of the *Bal*31-treated DNA (0.15 μg/μl) was ligated with T4 ligase. The ligated DNA was extracted with phenol and $CHCl_3$-isoamyl alcohol, ethanol precipitated, and centrifuged. The dried DNA pellet was resuspended in *Sal*I buffer and redigested with *Sal*I. The DNA was then transformed into $CaCl_2$-shocked *E. coli* HB101, and the resulting colonies were screened by the minilysate technique.

Restriction enzyme analysis of plasmids. Plasmids containing wild-type P160 genome or the deletion mutants were sized by double digests, using restriction enzymes *Sst*I and *Hin*dIII or *Bgl*II and *Sst*I (Bethesda Research Laboratories, Inc.). Standard reaction mixtures of 50 μl were stopped by adding 5 μl of sample buffer (20% [wt/vol] Ficoll, 20% [vol/vol] glycerol, 10 mM EDTA, 0.05% [wt/vol] bromophenol blue). Samples were loaded onto 6% polyacrylamide gels and run with TBE buffer (50 mM Tris-hydrochloride, 50 mM borate, 1 mM EDTA). Gels were run at 100 V for 5 to 7 h.

Transfection procedure. Plasmids containing A-MuLV genome inserts were prepared for transfection by *Hin*dIII (Bethesda Research Laboratories, Inc.) digestion followed by ligation at 15°C by T4 DNA ligase (100 to 200 μg of DNA per ml, 50 mM Tris [pH 7.5], 10 mM dithiothreitol, 5 mM $MgCl_2$, 0.8 mM ATP). *Hin*dIII digestion and ligation resulted in variable ligation products, some of which were tandem A-MuLV genomes. This arrangement provided for con-

secutive long terminal repeats necessary for virus spread (8). pZAP (pBR322 with the M-MuLV genome as insert) was prepared for transfection by ethanol precipitation and resolubilization in sterile TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA). Cotransfection of pZAP provided the helper virus proteins needed for propagation of A-MuLV. Salmon sperm carrier DNA was prepared by solubilizing overnight in TE (10 mM Tris [pH 7.5], 1 mM EDTA) followed by extraction with Tris-buffered phenol (pH 7.0), extraction with $CHCl_3$-isoamyl alcohol (24:1, vol/vol), and ethanol precipitation. DNA was resolubilized in sterile TE and frozen at −20°C.

Transfection onto NIH 3T3 cells was carried out by a modification of the calcium phosphate-DNA precipitate method of Graham and van der Eb (10). Cells were trypsinized 2 days before transfection and seeded onto 60-mm dishes at 10^5 cells per plate. The transfection cocktail was prepared by adding 10 μg of salmon sperm DNA to 0.5 ml of HEBS buffer (21 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 6.9, 150 mM NaCl, 0.7 mM Na_2HPO_4). A 2.5 M $CaCl_2$ concentration was added in three aliquots to a final concentration of 125 mM. After 20 min at room temperature, the cocktail was added to cells from which the media had been aspirated and left at room temperature for 20 min. A 2.5-ml portion of 37°C media (Dulbecco minimal essential media, 10% calf serum, penicillin, and streptomycin) was added, and the cells were incubated at 37°C. After 4 h, the DNA suspension was removed and 4.0 ml of 37°C media with 8 μg of Polybrene per ml was added to each plate. Plates were incubated for 2 h at 37°C and then refed with fresh media. Cells from each 60-mm plate were trypsinized 24 h later and plated onto two 100-mm plates. All plates were refed at 4-day intervals.

Mapping of integrated proviral genomes. Genomic DNA was prepared by the method of Steffen and Weinberg (23). Washed cell pellets (1×10^7 to 3×10^7 cells) were resuspended in 10 ml of room-temperature TNE buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA). Equal volumes of TNE plus 1% sodium dodecyl sulfate (SDS) were added to lyse cells. Proteinase K (200 μg/ml) was added and the mixture was incubated at 37°C overnight. The DNA was extracted twice with buffered phenol (pH 7.0) and three times with chloroform-isoamyl alcohol (24:1, vol/vol). Sodium acetate was added to 0.2 M, and the DNA was precipitated with 3 volumes of −20°C isopropanol. DNA was collected on a glass rod, left in vacuum overnight, and then resuspended in TE at concentrations of 200 to 400 μg/ml.

Restriction digests with *Kpn*I were carried out at 37°C with three- to fivefold enzyme excess. *Kpn*I digests included adenovirus 2 DNA as an internal control for complete digestion. The reactions (10 μg of DNA each) were run on 0.8% agarose gels, using TBE buffer (50 mM Tris, 50 mM borate, 1 mM EDTA). The gel was washed with 0.25 M HCl, denatured in alkali (0.5 M NaOH, 1.5 M NaCl), washed in neutralizing buffer (0.5 M Tris [pH 7.4], 3.0 M NaCl), and transferred to nitrocellulose (Schleicher & Schuell) with 20× SSC (1× SSC = 0.015 M sodium citrate and 0.15 M NaCl) by the method of Southern (21). The filters were prehybridized in prehybridization buffer (5× SSC, 5× Denhardt solution [1× = 0.1% bovine serum albumin, 0.1% Ficoll, and 0.1% polyvinylpyrollidine], 20 mM Na_2HPO_4 [pH 6.5], 100 μg of denatured salmon sperm DNA per ml) and hybridized with pAB3sub3, an A-MuLV-specific probe (7), in hybridization buffer (same as prehybridization buffer plus 10% [wt/vol] dextran sulfate) for 16 h at 65°C. The filters were washed three times in 2× SSC–0.1% SDS at 65°C. The filters were dried and autoradiographed, using intensifying screens (Dupont Cronex).

[^{3}H]leucine labeling. Exponentially growing cells were washed with phosphate-buffered saline twice and then resuspended in Dulbecco minimal essential medium minus leucine at a final cell concentration of 10^7 cells per ml. [^{3}H]leucine (100 μCi) was added and the cells were incubated at 37°C for 2 h. The cells were then washed in phosphate-buffered saline and lysed in PLB (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.01 M NaH_2PO_4-Na_2HPO_4 [pH 7.5], 0.1 M NaCl). Phenylmethylsulfonyl fluoride was added to a final concentration of 5 mM, and the lysate was clarified by centrifugation. Lysates were immunoprecipitated overnight (27). Samples were collected on *Staphylococcus aureus* Cowan strain (11), washed with PLB, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (2).

In vitro kinase assay. Cells were washed with phosphate-buffered saline and lysed in PLB at cell concentrations of 1×10^6 to 2×10^6 cells per ml. Lysates were clarified by centrifugating at 35,000 rpm for 2 h in the presence of 5 mM phenylmethylsulfonyl fluoride. Aliquots, 1 ml, were immunoprecipitated with 10 μl of anti-P15–anti-P12 (1:1) monoclonal antibodies (3) overnight at 4°C. The precipitates were then collected on *S. aureus*, washed once with PLB and once with 50 mM Tris-hydrochloride (pH 8.0), and resuspended in 50 μl of 50 mM Tris-hydrochloride (pH 8.0)–10 mM $MnCl_2$–1 μCi of [γ-^{32}P]ATP (24). The kinase reaction was incubated at 30°C for 5 min and stopped by washing with 1 ml of PLB. Samples were resuspended in 40 μl of sample buffer (50 mM Tris-hydrochloride [pH 6.8], 10% glycerol, 1% SDS, 1% 2-mercaptoethanol, 0.05% bromophenol blue), and 20 μl of each sample was electrophoresed on SDS–9% polyacrylamide gels and developed by autoradiography.

Two-dimensional phosphopeptide analysis of viral proteins. Approximately 5×10^7 A-MuLV-transformed cells were labeled in vivo with 2 mCi of $^{32}P_i$ by incubation in 5 ml of phosphate-free media with 10% dialyzed calf serum for 3 h at 37°C. Cells were washed with phosphate-buffered saline, lysed with PLB (10^7 cells per ml), and clarified by centrifugation. Viral proteins were immunoprecipitated with an anti-p15–p12 mixture, collected with *S. aureus*, and electrophoresed on SDS-polyacrylamide gels. Viral protein bands were visualized by autoradiography, eluted from dried gels, and prepared for analysis as described by Ponticelli et al. (15). Samples were loaded onto cellulose thin-layer plates and separated in the first dimension by electrophoresis in 1% ammonium carbonate (pH 8.9). Plates were dried and samples were separated in the second dimension by ascending chromatography in *n*-butanol–pyridine–acetic acid–water (15:10:3:12). Plates were dried and autoradiographed with Dupont Cronex intensifying screens.

RESULTS

Construction of deletion mutants in region IV of the A-MuLV P160 genome. The A-MuLV P160

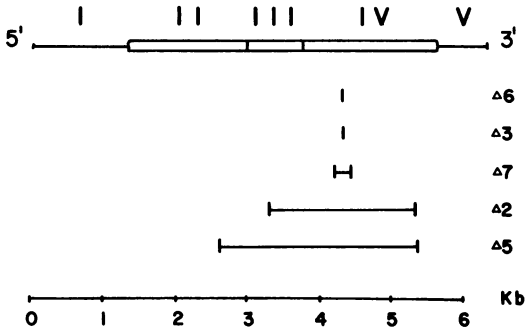

FIG. 1. Structure of the wild-type P160 genome and its deletion mutations. The wild-type P160 genome is shown divided into five regions. Two of the regions (I and V) are homologous with M-MuLV and three (II, III, IV) are unique to A-MuLV. Region II contains the kinase-essential region, region III is the P160-unique region, and region IV encodes the lethal sequences. The sequences deleted from region II, III, or IV are shown for each mutant listed in Table 1, determined by double-digest restriction maps as described in the text. Kb, Kilobases.

genome can be divided into five regions (Fig. 1). Regions I and V are, respectively, the 5' and 3' regions of the A-MuLV genome which contain M-MuLV sequences. Region II contains sequences necessary for in vitro kinase and transformation. P92td, a transformation- and kinase-negative virus, has a deletion which maps in this region. Region III contains the P160-specific sequences which have been deleted in phase in P120, the other wild-type A-MuLV strain. Region IV contains sequences from the 3' end of region III to the M-MuLV sequences at the 3' end of the genome. Large deletions of region IV enable P160-derived mutants to maintain stable transformation on fibroblast cell lines. These mutants express transforming efficiencies and in vitro kinase levels similar to those of the wild type (29). To alter the A-MuLV-transforming protein in region IV, we have prepared site-directed deletions of various sizes on cloned DNA (see Fig. 1).

The intact A-MuLV (P160) genome was inserted into the *Hin*dIII site of pBR322Δ*Sal*I, a vector which has been modified by deleting the *Sal*I site from pBR322 as described in Materials and Methods. This plasmid insert, therefore, contains a unique *Sal*I site located in region IV of the A-MuLV genome. By restricting with *Sal*I (Fig. 2) and digesting with *Bal*31 (a single-stranded endonuclease and double-stranded exonuclease [12]), plasmids with variable sized deletions in regions II, III, and IV were created by different lengths of digestion. The DNA was then transformed into $CaCl_2$-shocked *E. coli* HB101 (13), and the resulting colonies were screened for deletion mutants by restriction analysis of plasmid DNA made by a minilysate procedure.

By varying the time of digestion with *Bal*31, mutants with variable sized deletions were ob-

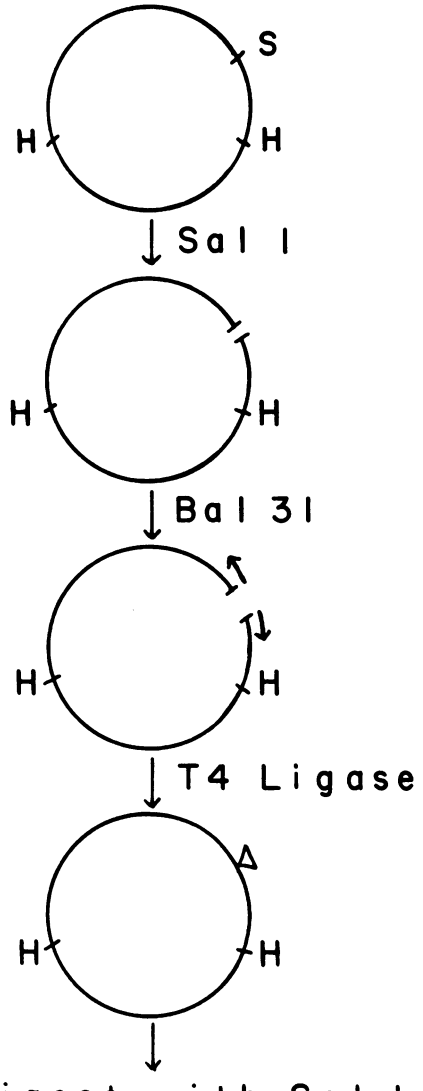

FIG. 2. In vitro mutagenesis of the wild-type P160 genome. The P160 genome was subcloned into the *Hin*dIII (H) site of pBR322Δ*Sal*I, a pBR322 vector lacking *Sal*I sites. *Sal*I linearizes the plasmid by cutting at a unique site in region IV of the A-MuLV genome. Deletions in region IV are created by digesting with *Bal*31. Plasmids are recirculized by T4 ligase and redigested with *Sal*I. *Sal*I redigestion reduces the efficiency with which nondeleted plasmids transform $CaCl_2$-shocked *E. coli*. Digestion and ligation conditions are described in the text.

TABLE 1. A-MuLV wild-type and mutant strains recovered after transfection into NIH 3T3 cells

Viral strain	Genome size (kb)[a]		Protein size (kd)		Lethal effect[c]
	Expected	Observed	Expected	Observed[b]	
P160	6.3	6.3	160	160	+
Δ6	6.3	ND[d]	130	160	ND
	6.3	ND	130	87	ND
Δ3	6.3	6.3	130	130	+
	6.3	ND	130	104	ND
	6.3	ND	130	102	−
	6.3	3.2	130	87	−
Δ7	6.1	ND	125	104	ND
Δ2	4.3	4.3	100	104	−
Δ5	3.3	ND	80	84	ND

[a] Expected genome sizes were determined by electrophoresing *Hin*dIII-digested plasmids on 0.8% agarose gels as shown in Fig. 2. Mutants with very small deletions (Δ3, Δ6, and Δ7) were further sized by electrophoresing *Bgl*II, *Sst*I, or *Hin*dIII-*Sst*I double digests on 6% polyacrylamide gels as described in the text. Observed genome sizes were determined through Southern blots as described in the text and shown in Fig. 6. kb, Kilobases.

[b] The viral protein sizes were measured by electrophoresing immunoprecipitated samples on SDS–9% polyacrylamide gels as shown in Fig. 4 and described in the text. Viral proteins P160, P106, and P85 were included as size markers. kd, Kilodaltons.

[c] Determinations of lethality were made by comparing the effects of virus infections on NIH 3T3 and BALB-1 cells as described in the text.

[d] ND, Not determined.

tained. Five mutants are shown in Fig. 3 and listed in Table 1. Figure 3A, lanes 1 through 6, shows *Hin*dIII digestions of A-MuLV wild-type (lane 1) and deletion (lanes 2 through 6) mutants. Digestion with *Hin*dIII releases pBR322 Δ*Sal*I and the A-MuLV genome, which migrates faster than the wild type depending on the size of the deletion (lanes 4 to 6). Deletion mutants in lanes 2 and 3 suffered deletions too small to be detectable with *Hin*dIII digestion but are detectable as a loss of the *Sal*I restriction site. Figure 3B, lanes 1 through 6, are double digests with *Hin*dIII and *Sal*I on plasmids containing A-MuLV wild-type (lane 1) and deletion (lanes 2 through 6) mutants. Only the plasmid containing wild-type A-MuLV is restrictable by *Sal*I, as evidenced by a shift in the mobility of the wild-type A-MuLV genome fragment, but not in the genomes of the deletion mutants. The smaller *Sal*I-*Hin*dIII fragment (1.2 kilobases) was electrophoresed off the gel in this experiment to better resolve larger fragments.

Recovery of A-MuLV deletion mutants through transfection into NIH 3T3 cells. The A-MuLV mutants as shown in Fig. 3 and others as summarized in Fig. 1 and Table 1 were transfected into NIH 3T3 cells by using a modification of the Graham and van der Eb calcium-phosphate procedure (see Materials and Methods). A-MuLV-transformed foci were generally visible 7 days post-transfection. The A-MuLV proteins produced by individual foci were determined after expansion by analyzing the ^{32}P-labeled viral proteins from in vitro immunoprecipitate kinase reactions on SDS-polyacrylamide gels. What we had expected to recover after transfection were mutant proteins truncated near or at the site of DNA deletion or indistinguishable from wild type due to a very small in-phase deletion (for example, Δ6-P160). What we found, however, were a series of mutants producing transforming proteins much smaller than predicted. Table 1 lists expected weights of the transforming proteins and the actual molecular weights from the recovered viruses. This unexpected result does not appear to be due to an undetected mutation in the original wild-type A-MuLV genome for two reasons. First, transfection with wild-type clone P160 yields viruses which produce the P160 size protein; and second, repeated transfections with the same A-MuLV DNA preparation can yield viruses producing proteins of the predicted sizes (Table 1). The frequent recovery of proteins between 87,000 and 104,000 in molecular weight led us to suspect that the truncations were not occurring randomly throughout

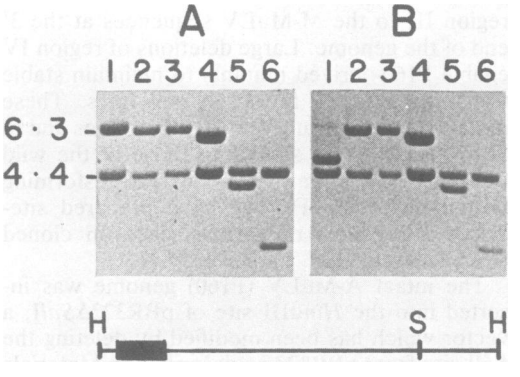

FIG. 3. Restriction digests of wild-type and mutagenized plasmids. *Hin*dIII digestions cut the viral genome inserts out of pBR322Δ*Sal*1 (A). Fragments shown are digests of P160 (lane 1), Δ6 (lane 2), Δ3 (lane 3), Δ7 (lane 4), Δ2 (lane 5), and Δ5 (lane 6). The sizes of the viral genome inserts are listed in Table 1. Further digestion by *Sal*I changes the mobility of the wild-type but not the deletion mutants (B). Fragments shown are from *Hin*dIII-*Sal*I double digests of P160 (lane 1), Δ6 (lane 2), Δ3 (lane 3), Δ7 (lane 4), Δ2 (lane 5), and Δ5 (lane 6).

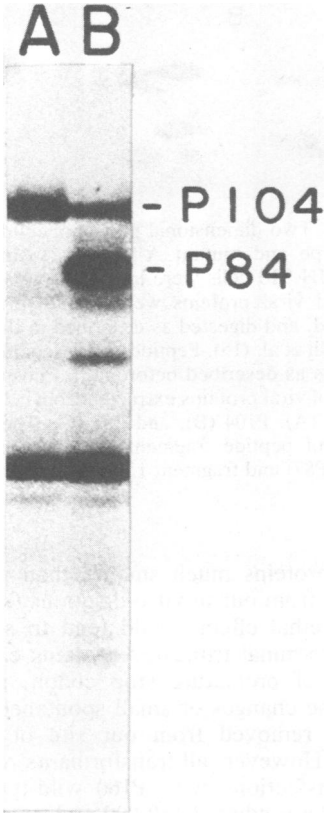

FIG. 4. Recovery of P84 through cotransfection. Transfections of Δ5 and Δ2 DNAs were carried out as described in the text. The transformed cells resulting from Δ2 and Δ5 cotransfections were screened as a mass population for expression of P84. These cells were then cloned, and individual clones were screened by in vitro kinase for expression of P84. The frequency of cells expressing P104 only (lane A) was approximately sevenfold higher than those expressing both P104 and P84 (lane B).

the protein but were a direct result of the lethal effect of the residual carboxy-terminal region of the transforming proteins. As a result, mutants which were predicted to lose 30,000 to 40,000 molecular weight from their carboxy terminus (a portion of region IV) expressed an unstable phenotype on DNA transfection and gave rise to much smaller proteins.

One large DNA deletion mutant we constructed (Δ5) was predicted to encode a carboxy-terminal, truncated, 80,000-molecular-weight protein. This would have removed sequences close to, but 3′ to, those already shown to be required for kinase and transforming activity (8, 25). DNA from Δ5 was not able to transform NIH 3T3 cells when analyzed by direct DNA transfections in multiple experiments. This mutant would thus establish the 3′ border of the kinase transformation-essential sequences if we could demonstrate that Δ5 DNA was transfectable and expressed the predicted A-MuLV protein of 80,000 molecular weight. We took advantage of the observation that cotransfected DNAs are frequently taken up by the same cells (14) and that different A-MuLV proteins can *trans* kinase each other as artificial substrates in the in vitro kinase reaction (28). We singly or cotransfected Δ5 (predicted to encode P80) with Δ2 (predicted to encode P104 already shown to be kinase transformation competent and nonlethal) onto NIH 3T3 cells. Transformed foci were obtained from Δ2 alone or Δ5 plus Δ2 and then expanded and extracted for the immunoprecipitate kinase assay as described in Materials and Methods. Many clones (even from the cotransformation) showed only the P104-sized protein (Fig. 4, lane A). An additional protein of 84 kilodaltons was recovered in clones derived only from the cotransformation (Fig. 4, lane B). Thus, the Δ5 DNA was transfectable and expressed a protein. The inability of P84 to transform NIH 3T3 cells by DNA transfection strongly suggests that the 3′ border of the transformation-essential region has been deleted or impinged upon in this mutant.

Protein analysis of A-MuLV mutants. Each mutant constructed has been recovered via DNA transfection onto NIH 3T3 cells, and cellular clones have been obtained by the microwell limiting-dilution technique. Each cloned mutant has been analyzed for the size of its A-MuLV protein estimated by SDS-polyacrylamide gel electrophoresis after immunoprecipitation of [^{3}H]leucine-labeled cellular lysates with an anti-*gag* gene reactive antibody (Fig. 5A).

P160 wild type (lane 1) is compared with P130 (lane 2), P104 (lane 3), P87 (lane 4), and a P84-P104 mixture (lane 5). Each A-MuLV protein is expressed as a doublet. The upper band of each doublet represents a carbohydrate-modified form of the protein. All of our current data suggest that this carbohydrate modification is controlled by the amino-terminal *gag* gene sequences in a fashion identical to those used in the parent M-MuLV virus (5, 20; O. Witte, C. Whitlock, F. Ponticelli, and D. Robertson, unpublished data). The gap between the upper and lower components of each doublet is more easily seen in the mutants with progressively larger truncations and lower apparent molecular weights. Only the lower band of the doublet for each mutant was labeled in the in vitro kinase reaction (Fig. 5B) (29). We have detected no major difference for kinase activity as monitored in this assay for P130, P104, or P87 compared with P160. However, with tyrosine-containing synthetic peptide substrates, much higher kinase efficiency for P160 was observed compared with

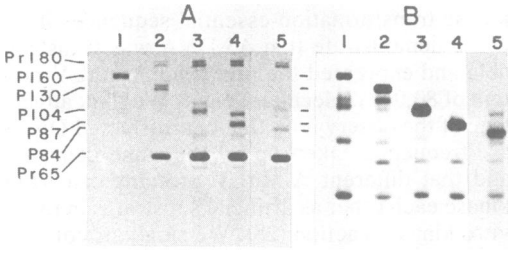

FIG. 5. Metabolic labeling and in vitro kinase activity of wild-type and mutant strains. A-MuLV-infected fibroblasts were labeled with [^{3}H]leucine and cell extracts were prepared as described in the text. Viral proteins were immunoprecipitated with antisera directed against P15 and P12. Samples were assayed on SDS–9% polyacrylamide gels for metabolically labeled protein (A) and in vitro kinase activity (B). (A) contains [^{3}H]leucine proteins from P160 wild type (lane 1), P130 (lane 2), P104 (lane 3), P87 (lane 4), and a P84-P104 mixture (lane 5). (B) Relative kinase levels of P160 (lane 1), P130 (lane 2), P104 (lane 3), P87 (lane 4), and a P84-P104 mixture (lane 5). Bands not labeled in (B) are due to degradation products of the intact A-MuLV proteins and to a nonviral phosphorylated species (26). In each numbered section control serum (left-hand lane) is compared with immune serum (right-hand lane); at these exposure times no bands were seen with control serum.

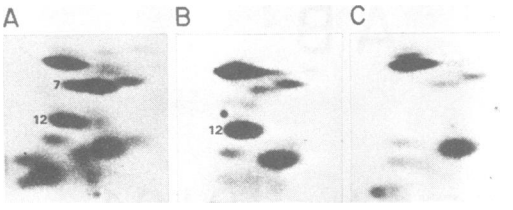

FIG. 6. Two-dimensional phosphopeptide analysis of wild-type and mutant A-MuLV. A-MuLV-transformed NIH 3T3 cells were labeled in vivo with $^{32}P_i$. ^{32}P-labeled viral proteins were immunoprecipitated, gel purified, and digested as described in the text and in Ponticelli et al. (15). Peptides were separated in two dimensions as described before (15). Phosphopeptide maps are of viral proteins extracted from cells infected with P160 (A), P104 (B), and P87 (C). The expected positions of peptide fragment 7 (deleted from both P104 and P87) and fragment 12 (deleted from P87) are noted.

P87 and P104 (J. Konopka and O. Witte, unpublished data).

We suspect that P84 is actually kinase negative but is labeled in trans by the cotransfected P104. Only after P84 is cloned away from other A-MuLV strains can we answer this unambiguously.

Further confirmation of the deletion of carboxy-terminal protein sequences in some mutants was shown by the loss of a specific phosphopeptide previously mapped to this region (Fig. 6; 15). P160, P104, and P87 were labeled in vivo with $^{32}PO_4$, isolated by immunoprecipitation, digested with trypsin, and then analyzed by thin-layer electrophoresis and chromatography (Fig. 6). Each protein showed a similar pattern of phosphopeptides except for the loss of peptide 7 in the P87 and P104 strains. This peptide has been peviously mapped to the last 20,000-molecular-weight segment of the carboxy terminus of the A-MuLV protein (15). An additional loss of peptide 12 has occurred in P87. Both peptides 7 and 12 are phosphorylated on serine residues. Mutant P130 has a phosphopeptide map indistinguishable from that of P160 (data not shown).

Analysis of integrated viral DNAs shows an unexpected large deletion as one cause of premature protein truncation. A number of our site-directed mutants recovered by transfection expressed proteins much smaller than those we predicted from our in vitro deletions (see Table 1). The lethal effect would tend to select for carboxy-terminal truncated proteins caused by any sort of premature stop codon, including single base changes or small spontaneous deletions far removed from our site of in vitro deletion. However, all transformants recovered from transfections with P160 wild-type DNA showed the predicted 160,000-molecular-weight protein.

The size of the integrated genome for a number of our recovered mutants has been analyzed by the Southern blotting technique. High-molecular-weight cellular DNAs restricted with *Kpn*I, an enzyme which cuts once in the viral long terminal repeats, were electrophoresed and then transferred and hybridized with an *abl* region-specific probe to monitor the overall genome size (Fig. 7; see Table 1). Although several mutants shown, such as Δ3 producing P130 (lane C), have the predicted size genome, some such as Δ3, producing P87, have suffered large spontaneous deletions (lane E). P87 has a genome size of about 3.2 kilobases. This mutant is readily transferable in its viral form, contains a complete *gag* region, and has kinase- and fibroblast-transforming activities (data not shown). All of this suggests that almost half of the P160 genome can be deleted without losing any function essential for transformation. Further restriction mapping of the Δ3(P87) genome has shown that the spontaneous deletion is confined to the 3′ half of the genome (S. Ziegler, S. Watanabe, and O. Witte, unpublished data). The possible role of our site-directed in vitro deletions in increasing the frequency of the larger spontaneous deletions recovered from transfections is not clear.

Abrogation of the lethal effect requires removal

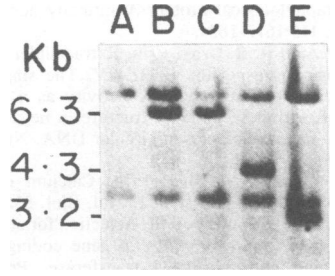

FIG. 7. Integrated genome sizes of wild-type and mutant A-MuLV. *Kpn*I (an enzyme that cuts in the viral long terminal repeat) was used to digest high-molecular-weight DNAs extracted from uninfected BALB/c thymus cells (lane A), P160 (lane B), P130 (lane C), P104 (lane D), and P87 (lane E). Digestions were electrophoresed on 0.8% agarose gels and transferred to nitrocellulose filters as described in the text. The filters were probed with the 2.3-kilobase (kb) *Bgl*II fragment from P160, an *abl*-specific probe. Hybridized fragments were detected by autoradiography, using Dupont Cronex intensifying screens.

of all of region IV from the A-MuLV protein. Although the recovery of small spontaneously deleted genomes from transfections (as above) is an indication of the lethal effect, our most sensitive assay is the response of BALB-1 cells to A-MuLV infection (29). A-MuLV stocks of wild-type and mutant strains rescued with M-MuLV helper virus were used to infect monolayers of BALB-1 cells at a multiplicity of infection sufficient to generate near-confluent transformation by 48 h. Equivalent aliquots of each population were passaged every 4 to 5 days and monitored visually for cell viability and percentage of transformed cells, as well as metabolic labeling or kinase activity, to follow the A-MuLV protein expression.

Wild-type P160 and mutant Δ3(P130) both showed the rapid conversion from transformed to flat phenotype with loss of A-MuLV expression by 7 to 10 days and are noted as positive for lethal effect (Table 1). Other mutants, Δ2(P104), Δ3(P103), and Δ3(P87), were able to maintain their expression and transformed phenotype for longer than 4 to 5 weeks in several experiments and are called negative for lethal effect (Table 1).

This assay is at best semiquantitative and lengthy, but taken together with the DNA transfection results (Table 1) it gives a consistent picture. Only those strains still expressing a major part of region IV continue to have a deleterious effect on BALB-1 or NIH 3T3 cells. From our previous work (29) the P120 wild-type strain (which has deleted region III but retains all of region IV) is still rapidly lethal to BALB-1 cells. Thus, the loss of expression of some or all of region III in Δ2(P104) and Δ3(P87) is probably not crucial for their decreased lethal phenotypes.

DISCUSSION

Although we are far from understanding the precise mechanism used by the Abelson virus to mediate a lethal effect, our studies suggest that two separate genetic regions within the same protein act synergistically. First, an active kinase function is required, as evidenced by lack of a lethal response in any of our kinase-negative mutants (29; unpublished data). Second, all of the lethal-minus mutant strains we have isolated, either biologically or by recombinant DNA techniques, show loss of expression of the carboxy-terminal region of the A-MuLV protein while still retaining kinase and transforming activities.

The frequent recovery of more abruptly truncated A-MuLV proteins, ranging from 87,000 to 105,000 in molecular weight, from site-directed deletions predicted to generate proteins of about 130,000 molecular weight suggests that expression of only a part of the sequences of region IV is required to mediate the lethal effect. Presumably, the high copy number of A-MuLV DNA taken up by recipient cells in the NIH 3T3 population increases the efficiency of the lethal effect (8). Other workers, using different A-MuLV clones, have not observed this lethal effect (22). It is interesting to note that we have not observed a high frequency of spontaneous truncation mutants arising from transfection of NIH 3T3 cells by the parent P160 DNA. Only when deletions are introduced in vitro around the *Sal*I site (Fig. 2) do we recover a high frequency of the unpredicted further truncation-deletions (Table 1). We do not know the structural basis for this observation.

We have unsuccessfully attempted to complement for the lethal effect in P90, a weak lethal strain that is kinase positive, by coinfection with P92td, a kinase-negative strain that has a complete region IV (29; unpublished data). This suggests that the kinase activity and the carboxy-terminal region function in *cis*. Several models are possible. Perhaps the carboxy-terminal region represents a regulatory domain which influences either the rate of kinase function or substrate selectivity. Interestingly, the kinase-essential sequences in region II and the carboxy-terminal region IV appear to be derived from separate exons of the c-*abl* gene and could represent unique structural domains (J. Wang, S. Goff, and D. Baltimore in reference 6). The availability of this series of A-MuLV mutant strains should aid in searching for such a regulatory function at an enzymatic level in future studies.

Finally, two of our new mutant strains have

helped to more precisely map the 3′ border of the transformation-essential sequences. The P84 strain does produce an A-MuLV-encoded protein but cannot morphologically transform NIH 3T3 cells during DNA transfection. The P87 strain is kinase positive, can transform fibroblasts efficiently, and retains a very low lymphoid-transforming activity. Although our protein size estimates based on electrophoretic migration are subject to some error, we can use them to approximate the coding region for the 3′ border of the transformation-essential region. This coupled with direct DNA sequence analysis will help to direct further mutagenesis attempts.

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