Abelson murine leukemia virus: Structural requirements for transforming gene function

(DNA transfection/plasmid pBR322/promoter/long terminal repeat)

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ABSTRACT   The integrated Abelson murine leukemia virus (A-MuLV) genome cloned in bacteriophage λgtWES·λB was used to localize viral genetic sequences required for transformation. Comparison of the biological activity of cloned A-MuLV genomic and subgenomic fragments showed that subgenomic clones that lacked the 5' long terminal repeat and adjoining sequences (300 base pairs downstream of the repeat) were not biologically active. In contrast, subgenomic clones that lacked the 3' long terminal repeat and as much as 1.3 kilobase pairs of the A-MuLV cell-derived abl gene were as efficient as wild-type viral DNA in transformation. The A-MuLV-encoded polyprotein P120 and its associated protein kinase activity were detected in transformants obtained by transfection with Cla I, BamHI, and HindIII subgenomic clones. In contrast, individual transformants obtained with subgenomic Sal I clones expressed A-MuLV proteins ranging in size from 82,000 to 95,000 daltons. Each demonstrated an associated protein kinase activity. These results provide direct genetic evidence that only the proximal 40% of abl with its associated 5' helper viral sequences is required for fibroblast transformation.

Abelson murine leukemia virus (A-MuLV) is a replication-defective transforming retrovirus that was isolated after inoculation of Moloney murine leukemia virus (M-MuLV) in prednisolone-treated BALB/c mice (1). A-MuLV induces B-cell lymphomas in vivo (2, 3) and is able to transform both lymphoid and fibroblastic cells in vitro (for review, see ref. 4). Several lines of evidence indicate that A-MuLV is a recombinant virus that contains 5'- and 3'-terminal portions of the M-MuLV genome joined to a segment of mouse cell-derived genetic information (5, 6). The latter has been designated abl according to recent convention (7).

A-MuLV transforming functions are thought to be mediated by the 120,000-dalton polyprotein, P120. This protein is a hybrid molecule containing M-MuLV gag gene structural proteins p15, p12, and a small part of p30, as well as a MuLV-unrelated component encoded by abl sequences (8). A number of naturally occurring A-MuLV variants, which make related proteins of larger and smaller sizes, have also been isolated (9).

Recent developments in recombinant DNA methodology and DNA transfection offer an important approach toward analysis of the functional organization of retroviral genomes. As an approach to study of the structural requirements for expression of the A-MuLV transforming gene, infectious integrated A-MuLV proviral DNA has recently been cloned in bacteriophage vector λgtWES·λB (6). In the present report, we describe the ability of A-MuLV subgenomic DNA fragments and subclones to transform cells in culture. Analysis of A-MuLV encoded proteins expressed by such transformants has made it possible to

localize and functionally characterize the region of abl required for transformation.

MATERIALS AND METHODS

Chemicals. T4 DNA ligase, DNA polymerase I, pBR322 DNA, and restriction endonucleases were purchased from New England BioLabs or Bethesda Research Laboratories. Restriction endonuclease reactions were carried out according to conditions provided by the suppliers.

Preparation of A-MuLV DNA Fragments and Subcloning in Plasmid pBR322. Recombinant phage (λAM-1) was grown, and DNA was isolated as described (6). The A-MuLV DNA insert was excised by EcoRI digestion and separated from the λ arms by preparative gel electrophoresis. DNA was recovered from the gel according to the method of Maxam and Gilbert (10). A-MuLV subgenomic fragments were prepared in the same manner after digesting the insert DNA with the appropriate restriction enzymes.

Plasmid pBR322 DNA was digested with a suitable restriction enzyme (depending on the restriction site to be used for cloning) and treated with bacterial alkaline phosphatase in an effort to reduce intramolecular ligation. Target and vector pBR322 DNAs were ligated by T4 DNA ligase and used to transform calcium-treated cells of Escherichia coli strain C600 (11). Transformants containing A-MuLV DNA fragments inserted into pBR322 DNA were detected by colony hybridization with nick-translated A-MuLV DNA as a probe (12).

Electron Microscopy. Equal amounts of DNAs (2–5 μg) were mixed, denatured with 30 mM NaOH, neutralized with 0.1 M Tris·HCl (pH 8.0), and allowed to hybridize in the presence of 50% formamide at 25°C for 4–6 hr. Samples were spread onto a distilled water hypophase and prepared for electron microscopy according to the method of Davis et al. (13). Uranyl acetate-stained grids were rotary shadowed with platinum/palladium and examined in a Siemens (101) electron microscope.

DNA Transfection. Transfection of NIH/3T3 cells with molecularly cloned DNA was carried out by the calcium phosphate precipitation technique (14) as modified by Wigler et al. (15). Briefly, 1 ml of 0.25 M $CaCl_2$ containing various amounts of test DNA and 50 μg of salmon sperm DNA as carrier was mixed with an equal volume of 50 mM Hepes, pH 7.1/280 mM NaCl/1.5 mM sodium phosphate. The calcium phosphate precipitate was placed in a 10-cm Petri dish in which $1–2 \times 10^5$ NIH/3T3 cells had been plated 24 hr earlier. After overnight incubation, the supernatant was removed, and 10 ml of Dulbecco's modified Eagle's medium/5% calf serum was added. Transformed foci were scored at 14–21 days.

Abbreviations: A-MuLV and M-MuLV, Abelson and Moloney murine leukemia virus, respectively; LTR, long terminal repeat; bp, base pair(s); kbp, kilobase pair(s).

Antisera. Goat anti-M-MuLV sera were obtained from Research Resources, Biological Carcinogenesis Branch, National Cancer Institute.

Cell Labeling and Immunoprecipitation. Subconfluent cultures were labeled with [^{35}S]methionine (120 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) at 0.05 mCi/ml for 3 hr in methionine-free Dulbecco's modified Eagle's medium. Radiolabeled cells were lysed and immunoprecipitated as described (16). Immunoprecipitates were analyzed by electrophoresis on a 7% polyacrylamide gel, and the gel was then fluorographed.

Assay for Protein Kinase Activity. The basic protocol of Collett and Erikson (17) was used. Immunoprecipitates bound to protein A-Sepharose beads were incubated with 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) at 30°C for 10 min in the presence of 0.025 ml of 20 mM Tris·HCl, pH 7.2/5 mM MnCl$_2$. ^{32}P$_i$-Labeled proteins were analyzed by electrophoresis on a 7.0% polyacrylamide gel followed by exposure to Kodak XR-5 film in the presence of intensifying screens.

RESULTS

Structure of the A-MuLV Genome. We have previously reported the cloning and characterization of infectious integrated A-MuLV DNA (6). Recombinant DNA clones containing a 7.8-kilobase-pair (kbp) EcoRI fragment were shown to include the entire integrated A-MuLV genome with 5' and 3' ends flanked by 1.8 kbp and 0.2 kbp of mink cellular DNA sequences, respectively (6). To map abl, A-MuLV DNA was annealed with a clone of integrated M-MuLV DNA. A representative heteroduplex structure (Fig. 1) shows that the viral genomes shared homologous sequences of about 1.7 and 0.7 kbp at their ends. By use of defined A-MuLV subgenomic DNA fragments in heteroduplex analysis, it was possible to orient the 1.7-kbp M-

MuLV homologous sequence at the 5' terminus of the A-MuLV genome (data not shown). Finally, in place of ≈6.6 kbp of DNA sequences deleted from M-MuLV DNA, A-MuLV DNA contained a large substitution (Fig. 1) defining the length of abl as 3.1 kbp.

Transformation by Cloned Whole and Subgenomic A-MuLV Fragments. To identify the minimal A-MuLV DNA sequences required for transformation, we monitored the biologic activity of λ AM-1 DNA after treatment with different restriction enzymes. As shown in Table 1, restriction enzymes Ava I, Sma I, Pst I, Sac I, Bgl I, and Bgl II, which cleave at multiple sites within the viral genome, completely abolished transforming activity. Both Xba I and Kpn I cleave the A-MuLV genome only within the long terminal repeat (LTR) (Fig. 2). Xba I significantly reduced and Kpn I completely abolished transforming activity. Pvu I, which cleaves at a unique site 300 kbp downstream from the 5' LTR, also abolished A-MuLV biologic activity (Table 1).

Cleavage with enzymes that cut at unique sites toward the 3' end of the viral genome made it possible to localize the region of abl required for transforming activity. Thus, BamHI, HindIII, and Cla I, which cut at unique sites toward the 3' end of the genome did not appreciably alter the biological activity of λ AM-1 DNA (Table 1). Even cleavage at a unique Sal I site within abl sequences resulted in transforming activity comparable with that of the parental A-MuLV DNA. In contrast, Acc I, which cleaves approximately 700 base pairs (bp) upstream from the Sal I site, completely abolished A-MuLV biological activity. These results suggested that not more than 56% of abl was required for fibroblast transformation.

Comparison of Transforming Activities of A-MuLV Subgenomic DNA Clones. In an effort to provide more quantitative comparisons of their transforming activities, we identified A-
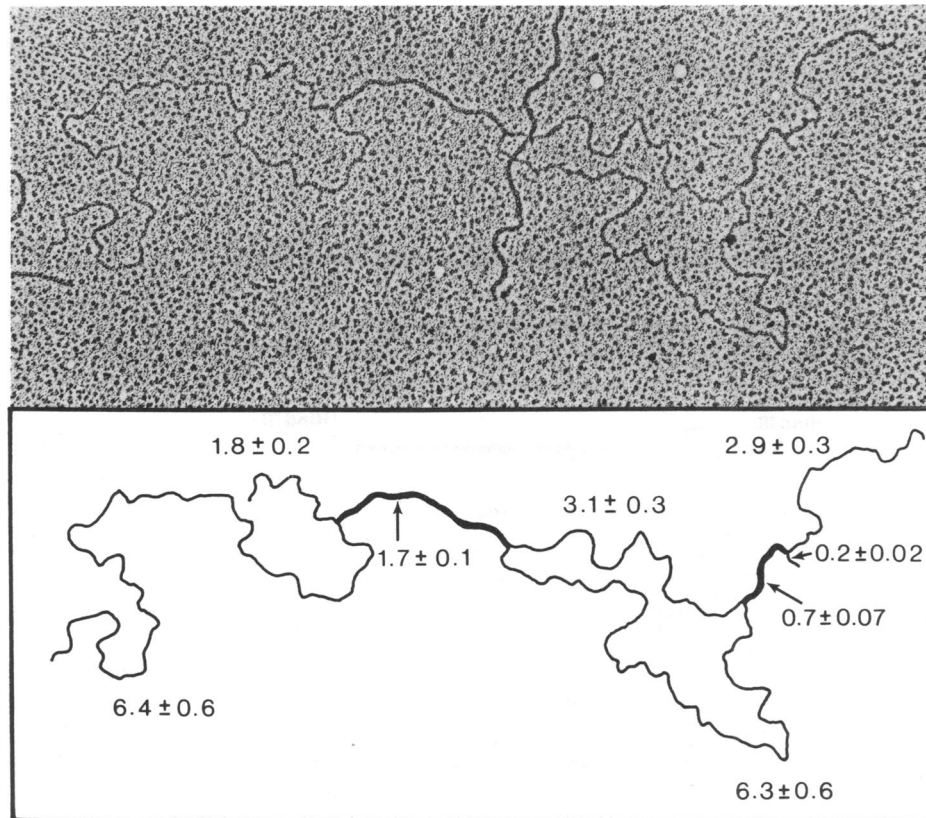

FIG. 1. Electron microscopy of a heteroduplex formed between A-MuLV and M-MuLV cloned proviral DNAs. A diagrammatic representation of the heteroduplex is shown below the electron micrograph. Contour lengths are given in kbp.

Table 1. Biological activity of cloned A-MuLV DNA after cleavage with restriction endonucleases

Endonuclease	Cleavages in genome, no.	Transforming activity
BamHI	1	>300
HindIII	1	>300
Cla I	1	>300
Sal I	1	>300
Pvu I	1	0,0
Acc I	2	0,0
Kpn I	2	0,0
Xba I	2	2,10
Sac I	3	0,0
Bgl I	3	0,0
Bgl II	3	0,0
Sma I	4	0,0
Pst I	5	0,0
Ava I	7	0,0

Bacteriophage λ containing A-MuLV proviral DNA (λ AM-1) was cleaved with various restriction enzymes and used to transfect NIH/3T3 cells. Transforming activity was determined by analysis of duplicate cultures by using 1:10 serial dilutions of each DNA preparation and is expressed as focus-forming units/μg of DNA. Focus formation was a linear function of DNA concentration.

MuLV DNA fragments to be used for subcloning in a plasmid vector (Fig. 2). In most cases, it was possible to use the same restriction sites in pBR322 for construction of the subclones. However, for Xba I, Acc I, and Kpn I subclones, it was necessary to use EcoRI linkers. As shown in Fig. 2, A-MuLV DNA subcloned in pBR322 had an infectivity of 1.7×10^2 focus-forming units/μg of DNA when used to transfect NIH/3T3 cells. Cla I, BamHI, HindIII, and Sal I DNA subclones showed comparable transforming efficiencies, while the Acc I subclone was completely inactive under the same assay conditions. These findings directly established that the distal 44% of abl was not essential for efficient transformation of fibroblasts.

Analysis of A-MuLV DNA subclones constructed from the

Pvu I-, Xba I-, and Kpn I- restricted wild-type genome provided information concerning the requirements for transformation of sequences at the 5'-terminal portion of the A-MuLV genome. Both Pvu I and Kpn I subclones lacked detectable biological activity. The transforming efficiency of the Xba I subclone was 3–10% that of wild-type A-MuLV DNA. These latter findings demonstrate that, while the 5' viral LTR is critical to transformation by A-MuLV, the entire LTR is not absolutely essential.

Identification of Gene Products Encoded by A-MuLV Subgenomic DNAs. A-MuLV is known to code for expression of P120, a M_r 120,000 protein, that contains the gag gene products p15, p12, and a small portion of p30, as well as a region unrelated to known MuLV proteins (4). Since transformation by A-MuLV is thought to be mediated by P120, it was of interest to analyze A-MuLV-specific proteins present in cells transformed by subgenomic A-MuLV DNAs. Cells were metabolically labeled with [^{35}S]methionine and viral proteins were analyzed by immunoprecipitation with anti-M-MuLV sera followed by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 3). At least three individual transformants of each DNA subclone were analyzed. Transformants induced by A-MuLV genomic DNA as well as by Cla I, BamHI, and HindIII subgenomic clones were found to express proteins whose sizes were indistinguishable from P120 encoded by wild-type A-MuLV (Fig. 3). These results indicate that the A-MuLV sequence coding for P120 terminates prior to the HindIII site. Transformants obtained with the Sal I DNA subclone demonstrated anti-M-MuLV-precipitable proteins whose sizes varied from 82,000 to 95,000 daltons among individual transformants tested (Fig. 3).

Previous studies have indicated that wild-type A-MuLV-encoded P120 possesses an associated protein kinase activity (18). Analysis of transformants induced by different A-MuLV DNA subclones in each case revealed A-MuLV-specific proteins having this activity (Fig. 4). Moreover, the sizes of the proteins corresponded to those of the A-MuLV-specific proteins precipitable with anti-MuLV serum in each transformant (Fig. 4). Protein kinase activity was even preserved in the Sal I subclone-encoded 82,000-dalton protein. These results help to localize
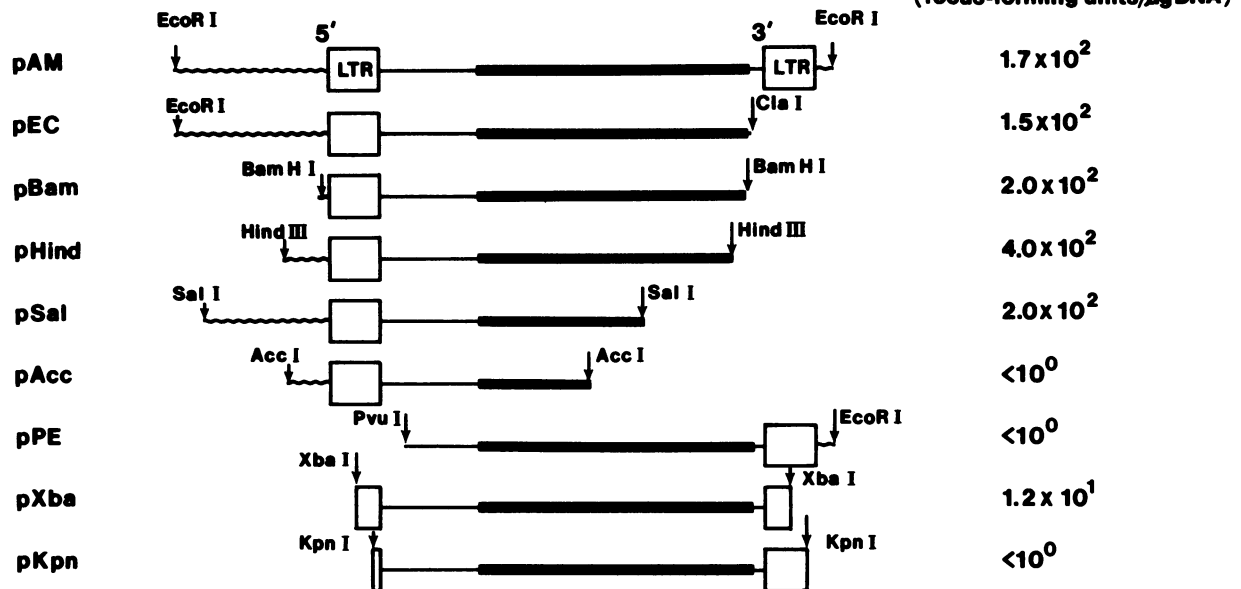

FIG. 2. Physical maps of genomic and subgenomic A-MuLV proviral DNAs cloned in plasmid pBR322. Fragments generated with Xba I, Kpn I, and Acc I were subcloned by using EcoRI linkers. Flanking host cellular DNA sequences are represented by wavy lines, and LTRs are indicated as rectangles. Heavy solid lines represent the A-MuLV-specific abl sequence. Recombinant plasmid DNAs were treated with the appropriate restriction endonuclease to release the insert before use in transfection experiments. Foci were scored at 14–21 days.
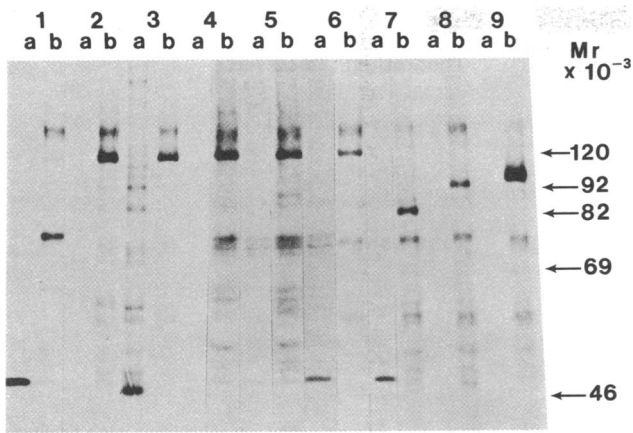
FIG. 3. Analysis of A-MuLV proteins expressed by cell lines transformed by cloned A-MuLV subgenomic DNAs. [³⁵S]Methionine-labeled proteins were extracted from cells, immunoprecipitated, and separated on a 7% polyacrylamide gel. Lanes: 1, uninfected NIH/3T3 cells; 2, an NIH/3T3 transformant with wild-type A-MuLV; 3–9, individual NIH/3T3 transformants obtained with various A-MuLV recombinant DNAs: pAM, pEC, pBam, pHind, pSal, pSal, and pSal. Goat preimmune (lanes a) and anti-M-MuLV (lanes b) sera were used to immunoprecipitate A-MuLV-specific proteins from the cell extracts.

the coding region for the A-MuLV transforming protein responsible for its kinase activity to sequences within the proximal 40% of *abl*.

DISCUSSION

A-MuLV is a prototype of transforming retroviruses that synthesize their transforming proteins by means of a *gag*-X polyprotein, the amino-terminal region of which is composed of helper virus *gag* gene products. Such transforming proteins appear to use helper viral *gag* gene sequences as initiators for
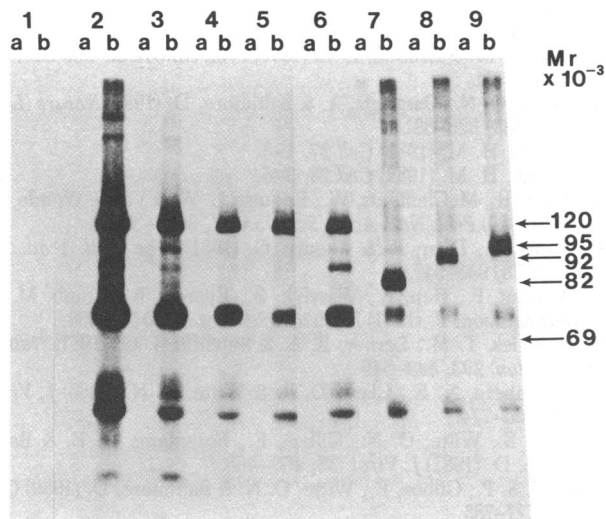

FIG. 4. Protein kinase activity associated with A-MuLV proviral DNA-transformed cell lines. Postmicrosomal fractions of cell lines transformed by A-MuLV recombinant DNAs (see Fig. 3) were incubated (final vol, 0.1 ml) at 0°C for 30 min with 5 μl of preimmune goat serum (lanes a) or goat anti-M-MuLV serum (lanes b). Immunocomplexes were mixed with 0.2 ml of protein A-Sepharose 4B beads (Pharmacia), collected by centrifugation, washed extensively, suspended in 25 μl of 20 mM Tris·HCl, pH 7.2/5 mM MnCl₂ containing 10 μCi of [γ-³²P]ATP (3,000 Ci/mmol), and incubated at 30°C for 10 min. Samples were analyzed by electrophoresis on 7% NaDodSO₄/polyacrylamide gels.

their synthesis. The availability of biologically active molecular clones of A-MuLV DNA made it possible to molecularly dissect the regions within the viral genome required for transformation. An important structural feature of the retroviral genome is the occurrence of LTRs at either end of the proviral genome (19). LTRs bear striking similarities to the terminal repeats of prokaryotic transposable elements and contain signals for initiation and termination of transcription (20, 21). Our analysis of A-MuLV subgenomic clones showed that the 3′ LTR was not essential for A-MuLV biological activity but that sequences in the 5′ LTR were crucial to efficient transformation.

Xba I cleaves the M-MuLV LTR at approximately the midpoint, retaining promoter and mRNA capping sites but deleting the 72-bp tandem repeat. In simian virus 40, a tandem 72-bp repeat has been reported to be essential for efficient transcription and tumor antigen synthesis (22, 23). Our findings indicated substantial reduction of transforming activity by the *Xba* I subclone. Whether this is due to loss of the tandem repeat or other sequences upstream from the *Xba* I site remains to be resolved. *Kpn* I, which deletes the promoter and mRNA cap site, as well as the tandem 72-bp repeat within the LTR, totally abolished A-MuLV transforming activity. These results provide *in vivo* demonstration of the functional nature of the putative control elements that reside in the retroviral LTR.

Comparison of the theoretical coding capacity of the gag-abl protein encoded by the wild-type A-MuLV genome with results of analysis of the sizes of the proteins expressed by subgenomic A-MuLV DNA clones led to several conclusions. The M-MuLV *gag* gene product is formed by processing of a precursor polypeptide whose size is approximately 6,000 daltons more than the 65,000-dalton polyprotein comprised of p15, p12, p30, and p10. It has not been established whether the gag-abl protein of A-MuLV is processed in an analogous manner. However, based on nucleotide sequence analysis of M-MuLV and A-MuLV (ref. 24; unpublished data) and immunologic analysis of the *gag* gene products present in the A-MuLV gag-abl protein (9), the *gag* gene-encoded protein must represent 30,000–36,000 daltons of this protein. *abl* itself has a theoretical coding capacity of approximately 115,000 daltons based on the results of nucleotide sequence analysis (unpublished observation) and heteroduplex analysis (Fig. 1). Thus, the wild-type viral genome could code for a gag-abl protein of 145,000–150,000 daltons if it used the entire extent of *abl* sequences (Fig. 5). The wild-type A-MuLV genome used by us codes for a protein of only 120,000 daltons. Our findings that transformants induced by *Hind*III, *Bam*HI, and *Cla* I subclones of this genome expressed proteins of 120,000 daltons demonstrate that coding sequences for P120 must terminate within *abl* sequences.

The theoretical coding capacity of the *Sal* I subgenomic A-MuLV DNA fragment is 95,000–100,000 daltons. However, we observed that *Sal* I transformants expressed A-MuLV-specific proteins with molecular weights ranging from 82,000 to 95,000 (Fig. 5). These results indicate that such proteins terminate at various points prior to the *Sal* I site. One possible explanation for the observed variations in sizes of proteins encoded by *Sal* I clones is the loss of DNA sequences from the termini of DNA molecules during the transfection process. The fact that this was not observed with either wild-type or the larger A-MuLV subgenomic clones analyzed may be explained by the fact that such clones contain several hundred nucleotides of noncoding *abl* sequences at their 3′ termini. Loss of these sequences obviously would not affect the coding region of the molecule.

A number of naturally occurring variants of A-MuLV have been isolated (8, 25). Variants that express proteins as small as 90,000 daltons have been shown to retain transforming activity for fibroblasts (25). While precise mapping data for these vari-
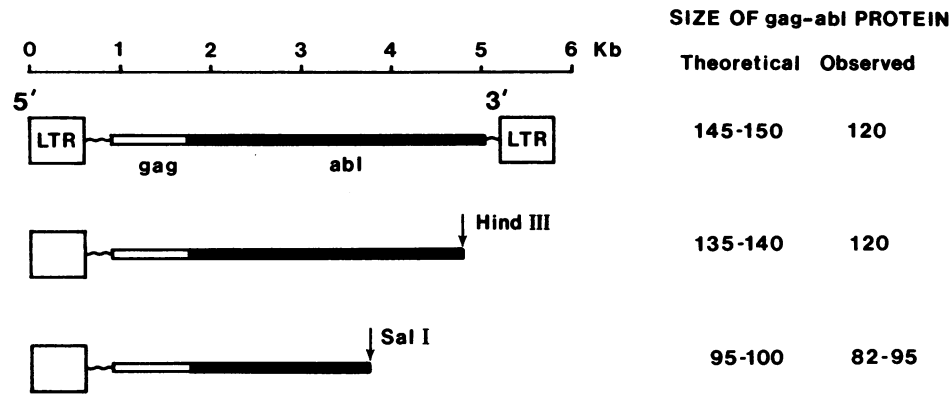
FIG. 5. Comparison of wild-type and subgenomic A-MuLV DNAs with respect to coding capacities and observed transforming gene products. A-MuLV-specific *abl* sequences are represented by solid lines. Theoretical sizes of the A-MuLV transforming gene products (in kilodaltons) are based on nucleotide sequence analysis of the A-MuLV genome (unpublished observations).

ants is not yet available, it has been presumed that they have arisen by mutations or small deletions leading to premature termination of P120 (26). Our *Sal* I subgenomic DNA transformant that expressed an 82,000-dalton protein represents the smallest known A-MuLV-encoded transforming protein. This protein would use only around 40% of *abl* in addition to the *gag* gene-encoded portion of the molecule.

A number of transforming retroviruses including A-MuLV have been shown to possess closely associated kinase activity with specificity for tyrosine phosphorylation (18). In the case of A-MuLV P120, the protein appears to be autophosphorylated at tyrosine sites within the molecule (18). In the present studies, even the smallest A-MuLV-encoded protein, that of 82,000 daltons, had an associated protein kinase activity. Thus, both activities are likely to be coded by the 5' portion of *abl*.

The mouse cell homologue of v-*abl*, designated c-*abl*, is a complex gene that has been reported to contain multiple intervening sequences (27). By restriction mapping, the distal 1.3 kbp of v-*abl* is colinear with one domain of c-*abl*. As shown here, the minimum coding region of v-*abl* required for its transforming function lacks this entire domain. These findings argue either that c-*abl* represents a single gene possessing more than one functional domain or that A-MuLV arose by recombinational events involving portions of more than one gene, only one of which is essential for its fibroblast-transforming activity. Studies of the transcription of c-*abl* in normal and transformed cells have revealed a complex pattern with multiple transcripts detected (28). Analysis of such transcripts by using 5' and 3' *abl*-specific probes generated in the present studies should help to resolve whether v-*abl* coding sequences are comprised of one or more than one genetic element.

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