Transfection of Fibroblasts by Cloned Abelson Murine Leukemia Virus DNA and Recovery of Transmissible Virus by Recombination with Helper Virus

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Received 17 August 1981/Accepted 6 October 1981

A cloned, permuted DNA copy of the Abelson murine leukemia virus (A-MuLV) genome was capable of eliciting the morphological transformation of NIH/3T3 fibroblasts when applied to cells in a calcium phosphate precipitate. The efficiency of the process was extremely low, yielding approximately one transformant per microgram of DNA under conditions which give 10^4 transfectants per microgram of other DNAs (e.g., Moloney sarcoma virus proviral DNA). The DNA was able to induce foci, even though the 3' end of the genome was not present. The transforming gene was thus localized to the 5' portion of the genome. The transformed cells all produced viral RNA and the virus-specific P90 protein. Transmissible virus could be rescued from these cells at very low frequencies by superinfection with helper virus; the rescued A-MuLV virus had variable 3' ends apparently derived by recombination with the helper. Dimerization of the permuted A-MuLV cloned genome to reconstruct a complete provirus did not improve transformation efficiency. Virus could be rescued from these transformants, however, at a high efficiency. Cotransfection of the permuted A-MuLV DNA with proviral M-MuLV DNA yielded a significant increase in the efficiency of transformation and cotransfection of dimeric A-MuLV and proviral M-MuLV resulted in a high-efficiency transformation yielding several thousand more transformants per microgram than A-MuLV DNA alone. We propose that helper virus efficiently rescues A-MuLV from transiently transfected cells which would not otherwise have grown into foci. We hypothesize that multiple copies of A-MuLV DNA introduced into cells by transfection are toxic to cells. In support of this hypothesis, we have shown that A-MuLV DNA sequences can inhibit the stable transformation of cells by other selectable DNAs.

Abelson murine leukemia virus (A-MuLV) is a replication-defective retrovirus which transforms immature lymphocytes of the B-cell lineage to cause a rapidly progressing lymphosarcoma (1). In vitro, A-MuLV can cause the transformation of primary fetal liver and bone marrow lymphoid cells (23) and the fibroblastic NIH/3T3 cell line (26). The virus was apparently derived by recombination between the parent Moloney murine leukemia virus (M-MuLV) and a cellular gene, termed *C-abl*, normally expressed in lymphoid cells (9, 11, 27).

The A-MuLV genome consists of a central substitution of cellular DNA (*V-abl*) bracketed by regions of homology to the M-MuLV genome. The hybrid genome encodes a single known protein, which is thought to be responsi-

ble for the novel transforming activity of the virus (21, 38). The protein contains antigenic determinants of the parent M-MuLV *gag* product and new determinants encoded by the *V-abl* region. Associated with the P120 protein is a protein kinase activity, which transfers the gamma phosphate of ATP onto tyrosine residues in the Abelson protein itself (35). Viral mutants which reduce or abolish this kinase activity also reduce or abolish the ability to transform cells (24, 36).

We have previously prepared molecular clones of DNA copies of the A-MuLV genome and have used these clones to analyze the structure of the viral DNA and the relationship between the *V-abl* region and its cellular homolog, *C-abl* (9). Analysis of the functions of the different parts of the A-MuLV genome, however, requires an assay for the biological activity of these cloned DNAs. In this paper, we describe

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the successful transfection of these cloned DNAs into NIH/3T3 cells to yield transformed foci. The efficiency of A-MuLV DNA transfection was surprisingly low and could be increased by procedures that facilitated rescue of virus from the initially transfected cell and infection of surrounding cells. A portion of the viral genome at the 3' end was found to be dispensable for transforming activity. Proviruses lacking the 3' end of the genome still directed the synthesis of viral RNA and proteins. These defective genomes could be rescued from cells by superinfection with helper virus; the recovered viruses were recombinants between the A-MuLV provirus and the helper. In this way, a series of new A-MuLV genomes could be isolated containing variable amounts of helper sequences at the 3' end.

MATERIALS AND METHODS

Cells, virus, and DNAs. NIH/3T3 fibroblasts were grown in Dulbecco modified Eagle medium with 10% calf serum and were maintained as described elsewhere (2). M-MuLV was harvested from clone 1 cells constitutively producing virus (8). M-MuLV titers were determined by XC plaque assay (25), and A-MuLV titers were determined by focus formation on NIH/3T3 cells (26). Infection of cells by M-MuLV to yield producer cells was carried out in the presence of 8 μ g of Polybrene per ml for 2 h at 37°C; virus was harvested from producer cells after 12 h. Labeled virion RNA was prepared and analyzed as described elsewhere (28).

Clone λ Ab1 and the subclone pAB3Sub3 were as described (9). pSV2GPT DNA was a gift of R. Mulligan. Plasmid pAb1Ab1 was prepared by cleavage of λ Ab1 DNA with *Hind*III and by ligation of the resulting mixture to *Hind*III-cleaved pBR322 DNA (7). The product was used to transform *Escherichia coli* to ampicillin resistance, and plasmid DNA was analyzed (20) for the presence of A-MuLV DNA.

Analysis of DNA, RNA, and proteins. The preparation of total cellular DNA, restriction enzyme digestions, gel electrophoresis, and transfer to nitrocellulose (30) were all as previously described (11). 32 P-labeled probes were prepared by nick translation (22).

Total cell RNA was prepared by phenol extraction of lysates made in the presence of the RNase inhibitor vanadyl adenosine (4). Polyadenylate-containing RNA was selected on oligodeoxythymidylate cellulose (3) and analyzed by electrophoresis through agarose gels after denaturation with glyoxal (17). The RNA was blotted to diazophenylthio paper and hybridized to labeled DNA probes as described elsewhere (32).

Proteins synthesized by infected cells were examined by immunoprecipitation of lysates of cells metabolically labeled with [35 S]methionine. The proteins were analyzed by gel electrophoresis and fluorography as described elsewhere (38). Sera used were goat anti-Moloney serum (34) and anti-Abelson tumor serum (37).

Transfection of DNAs. Transfection of A-MuLV DNA was essentially as described for transformation with tumor DNAs (2, 14). Precipitates of calcium phosphate and DNA were applied to cells for 4 to 6 h.

The cells were then trypsinized and replated at a 5:1 dilution. Cells were fed every 5 days, and foci were counted after 15 to 20 days. Cells transformed by pSV2GPT DNA (18) were plated 1 day after transformation in medium containing (in micrograms per milliliter): hypoxanthine, 15; aminopterin, 0.2; thymidine, 5; xanthine, 250; glutamine, 150; glycine, 5; and mycophenolic acid, 25 (19). Colonies were counted after 14 to 20 days.

RESULTS

Biological activity of clone λ Ab1. We have previously described the cloning of circular double-stranded DNA copies of the A-MuLV genome synthesized soon after infection of mouse cells (9). In those experiments, the DNA circles were cleaved with *Hind*III, and the resulting permuted genomes were joined to the λ phage vector Charon 21A. The clones were derived from a strain of A-MuLV encoding P90, a virus-specific transforming protein of 90,000 daltons. To study the infectivity of the cloned DNAs, they were cleaved with *Hind*III, ligated into polymers, and applied to sensitive NIH/3T3 cells in a calcium phosphate precipitate (2, 14). Even 100 ng of DNA in each of three experiments gave rise in toto to only a single focus (TFD-1). Under these conditions, cloned murine sarcoma virus DNA (6) gave roughly 10^4 foci per 100 ng of DNA (see Table 1). Thus, the A-MuLV DNA was many orders of magnitude less efficient at stable transformation of cells.

Other transfections were carried out with NIH/3T3 cells constitutively producing M-MuLV as recipient cells. One focus (TFC-1) was recovered from a total of three experiments (Table 1). Thus, the presence of helper virus in these cells did not improve the very low efficiency of the transfection process. A third focus (TFA-1) was recovered from transfection of these cells without cleavage of the insert from vector DNA; excision and polymerization of the A-MuLV inserts were apparently not required for expression of biological activity.

Analysis of DNA from transfected cells. To confirm that the rare foci recovered in the trans-

TABLE 1. Transformation efficiency of various DNAs^a

DNA	Treatment	Cells	Foci/100 ng
λ Ab1	Cleaved and ligated	NIH/3T3	~0.1
λ Ab1	Cleaved and ligated	NIH/3T3/Mo ^c	~0.1
λ Ab1		NIH/3T3/Mo	0.1
MSV ^b		NIH/3T3	~ 10^4

^a DNAs were applied to the indicated cells in a calcium phosphate precipitate, and the foci were counted. Numbers are the averages of 5 to 10 experiments.

^b MSV, Murine sarcoma virus.

^c NIH/3T3/Mo denotes NIH/3T3 cells chronically infected with Moloney murine leukemia virus.

fection experiments were authentic transformants, the cellular DNA from the transfectants and normal NIH/3T3 cells was tested for the presence of new A-MuLV sequences. Total cellular DNA was isolated, cleaved with several restriction enzymes, blotted onto nitrocellulose paper, and probed with an *abl*-specific probe (9). Normal NIH/3T3 cell DNA contains a large region of homology to this probe—the endogenous *C-abl* gene—which, after *Bam*HI, *Hind*III, or *Eco*RI digestion, gives rise to a variety of fragments (Fig. 1A and B, 3T3 lanes; endogenous bands are relatively faint because the gel was exposed to show the intensity of the bands in the transfected cells). Digests of the DNA from each transfected line with these enzymes revealed the *C-abl* fragments and in addition either one or two new fragments which labeled very intensely (Fig. 1A and B). Therefore, these foci contained new DNA homologous to the *abl* sequence. The enzymes used did not cleave within the region of the A-MuLV genome con-

tained in the *abl* probe. Thus, the number of new bands seen indicated directly the number of distinguishable copies of the A-MuLV genome introduced into the cells. The very high intensity of hybridization seen suggested that the new DNA was present at an amplified level of 5 to 10 copies per cell, as has been observed for other transfected DNAs (12).

Cleavage with the enzyme *Xba*I suggested that these new A-MuLV DNAs were not the result of conventional viral infection. *Xba*I cleaves within the long terminal repeat (LTR) sequences which bracket normal proviruses (9); thus, it releases from authentic proviruses a fragment whose length is characteristic of the A-MuLV genome. Analysis of the *Xba*I-cleaved DNA from the three foci revealed fragments of a size different from that of the 5.6-kilobase proviral DNA (Fig. 2). Similar results were obtained after cleavage with *Kpn*I and *Sac*I, enzymes which also cleave in the LTR sequences (9) (data not shown). We concluded that the A-MuLV

FIG. 1. Analysis of viral DNA in three cell lines transfected with cloned A-MuLV DNA. Total cellular DNAs from NIH/3T3 cells and three transformed cell lines were cleaved with the indicated restriction enzymes, fractionated by agarose gel electrophoresis, hybridized with labeled DNA probes, and exposed to X-ray film. (A and B) DNAs were probed with an A-MuLV-specific probe; transformed cells contained new viral DNA in addition to the endogenous *C-abl* sequences found in 3T3 cells. (C) Radioactivity was washed from the filter shown in (B), and the filter probed with a phage lambda DNA probe. The bands common to all lanes are due to trace amounts of *Eco*RI-cleaved lambda DNA added as marker after digestion of the cell DNA. These cell lines contain one and two lambda DNA-containing fragments. In no case are these fragments the same as the virus-containing fragments detected in B.

FIG. 2. Analysis of structure of proviral DNA in two cell lines transfected with cloned A-MuLV DNA. Cell DNA was cleaved first with *Xba*I to provide a fixed endpoint in the LTR sequence and then with a variety of enzymes recognizing sites further downstream in the viral genome. The viral fragments were detected by hybridization to the 32 P-labeled A-MuLV-specific DNA probe. A summary of the structures is given in Fig. 6.

DNA in these cells was apparently not bracketed by the two LTRs that flank normal proviral DNA. Cleavage with *Xba*I plus other enzymes revealed that sites in the 5' half of the A-MuLV genome were present (Fig. 2) but that sites in the 3' half were missing.

Further proof that the cells were transfected could be obtained by testing for the presence of vector phage lambda DNA in the cell DNA. Although no selection for the vector DNA was applied, mixed DNAs are commonly acquired by the same cell (33). Reprobing the same blot shown in Fig. 1A showed that lambda DNA fragments were indeed present in these cells (Fig. 1C; the common bands in the lanes were due to added λ phage DNA used as marker, whereas the individual bands were due to transfection). Any focus deriving from a viral contamination would be unlikely to carry lambda DNA.

Expression of viral gene products by transfected cells. To examine whether the transfected cells expressed A-MuLV gene products, cellular RNA and proteins were analyzed. Total cell RNA was isolated from cultures of two cell lines. This RNA was separated by electrophore-

sis through an agarose gel, transferred to diazotized paper, and hybridized with an *abl*-specific probe. Autoradiography revealed large amounts of viral RNA (Fig. 3). In one case (lane TFC-1), the RNA seemed heterogeneous in size even with shorter autoradiographic exposures, whereas in another (lane TFA-1), RNAs of two discrete sizes were detected. The amounts of virus-specific RNA in the transfected cells were as much as or greater than that found in virus-infected cells (lane ANN-1). No discrete RNA identical in size to that of authentic viral RNA was detected. Major bands both larger and smaller than viral RNA were found.

The transfected cells were analyzed for the presence of the A-MuLV-encoded P90 protein by metabolic labeling with [35 S]methionine and immunoprecipitation with sera specific for the *gag* determinants or with anti-Abelson tumor serum (37). All three cell lines produced a P90 protein (two are shown in Fig. 4). Those lines derived by transfection of M-MuLV producer cells also contained the expected helper proteins Pr80^{env} and Pr65^{gag}.

Recovery of transmissible virus. Because the viral DNA contained in these transfected cells

FIG. 3. Analysis of viral RNA in cells transfected with A-MuLV DNA. Equal amounts of RNA isolated from the virus-infected cell line ANN-1 and cells transfected with A-MuLV DNA were fractionated by electrophoresis, blotted to diazotized paper, and hybridized with a radioactive A-MuLV-specific probe.

was not a complete provirus bracketed by two LTRs, the viral RNA produced should not be transmissible by conventional mechanisms. Nevertheless a low but measurable titer of A-MuLV focus-forming units was produced by those transfected cells which contained helper virus. Supernatant culture fluid from these cells had approximately 10^6 XC PFU/ml, but had focus-forming titers of approximately 1 focus per ml (Table 2). Several of the rare secondary foci were picked from plates infected by the virus liberated from the primary transfected cells and were grown into large-scale cultures. The majority of these secondary cell lines released high titers of both helper M-MuLV and focus-forming A-MuLV (Table 2). Thus, during the rescue of A-MuLV from the primary focus to produce the secondary foci, some event had restored the transmissibility of the virus to its normally high level. These results were similar to the rescue of defective Harvey sarcoma virus reported by Goldfarb and Weinberg (13) and suggested that, as in the Harvey sarcoma virus system, restoration of transmissibility might have occurred by restoration of the missing 3' end of A-MuLV by recombination with the helper M-MuLV. A few secondary foci (A12, A14, and C6) were M-MuLV producers but were rather low-level producers of A-MuLV. These rare foci apparently did not contain easily transmissible proviruses.

Structure of rescued A-MuLV: isolates with new genomic sizes. To examine the structure of the rescued A-MuLV genomes, DNA was isolated from each of 23 secondary foci and the structure of their A-MuLV proviral DNA was determined. Total cellular DNA was cleaved with each of three enzymes—*Xba*I, *Kpn*I, and *Sac*I—and the cleaved DNAs were displayed by agarose gel electrophoresis and probed by hybridization with the *abl* probe as before. All of these enzymes cleave at known locations in the 5' portion of the A-MuLV genome. The sizes of the A-MuLV-containing DNA fragments produced by these enzymes thus defined the position of the new sites at the 3' end of the provirus. New sites for cleavage by *Hind*III and *Bam*HI were also determined by analyzing double digests with *Xba*I plus *Hind*III and with *Sac*I plus *Bam*HI, respectively. Examples of several of these analyses are shown in Fig. 5. The new sites at the 3' end of the A-MuLV proviruses align perfectly with known sites in M-MuLV for nearly all of the clones analyzed (20 of 25 proviruses). The position of these sites could thus be used to define the position and extent of the new M-MuLV sequences at the 3' end. The mapping data are summarized in Fig. 6.

The total sizes of the new, rescued genomes varied widely: genomes were found which were

FIG. 4. Analysis of viral proteins in cells transfected with A-MuLV DNA. Cells were metabolically labeled with [35 S]methionine and the virus-specific proteins extracted, immunoprecipitated with specific antisera, separated by polyacrylamide gel electrophoresis, and detected by fluorography. M: goat anti-Moloney serum was used. A: Abelson murine tumor regressor serum specific for the A-MuLV-encoded protein was used.

TABLE 2. Virus expression by primary transfectants and secondary transformants

Cell Line	Source ^a	RT ^b	M-MuLV proteins ^c	P90	Released focus-forming units per ml
Primary					
TFD-1	λ Ab1 $\rightarrow$ 3T3	—	—	+	0
TFA-1	λ Ab1 $\rightarrow$ 3T3/Mo (uncut)	+	+	+	0.5–1
TFC-1	λ Ab1 $\rightarrow$ 3T3/Mo (cut and ligated)	+	+	+	0.5–1
TFE-1	pAb1Ab1 $\rightarrow$ 3T3	—	—	+	0
TFE-1/M	Superinfection of TFE-1	+	+	+	$>10^4$
TFB-1	λ Ab1 + pZAP $\rightarrow$ 3T3	+	+	+	$>10^4$
TFB-2	λ Ab1 + pZAP $\rightarrow$ 3T3	+	+	+	$>10^4$
TFB-3	λ Ab1 + pZAP $\rightarrow$ 3T3	+	+ ^d	+	$>10^4$
TFB-4	λ Ab1 + pZAP $\rightarrow$ 3T3	+	+ ^d	+	$>10^4$
TFB-5	λ Ab1 + pZAP $\rightarrow$ 3T3	+	+ ^d	+	$>10^4$
Secondary					
A1	TFA-1	+	+	+	2×10^5
A3	TFA-1	+	+ ^d	+	2×10^4
A4	TFA-1	+	+	+	6×10^4
A5	TFA-1	+	+	+	4×10^4
A6	TFA-1	+	+	+	4×10^3
A8	TFA-1	+	+	+ ^e	2×10^3
A9	TFA-1	+	+	+	6×10^3
A11	TFA-1	+	+ ^d	+	3×10^3
A12	TFA-1	+	+ ^d	Very low	6×10^1
A14	TFA-1	Very low	+ ^d	+	1×10^2
C1	TFC-1	+	+ ^d	+	3×10^4
C2	TFC-1	Low	+	+	4×10^3
C3	TFC-1	+	+ ^d	+	2×10^5
C4	TFC-1	+	+ ^d	+	2×10^4
C5	TFC-1	+	+ ^d	+	4×10^4
C6	TFC-1	+	+	+	1×10^2
C7	TFC-1	+	+ ^d	+	2×10^3
C8	TFC-1	+	+	Very low	2×10^3 (late)
C9	TFC-1	+	+	+	5×10^3
C10	TFC-1	+	+	+	2×10^4
C12	TFC-1	+	+	+	1×10^4
C13	TFC-1	+	+	+	2×10^3
C14	TFC-1	+	+	+	5×10^3

^a The DNA or mixture of DNAs and the cell line used in the transformation are given.

^b The results of the rapid reverse transcriptase assay are given (11).

^c Cells containing Pr65^{gag} and Pr80^{env} intracellularly as determined by gel electrophoresis are denoted as +; those not expressing detectable levels of these proteins are denoted as —.

^d These cell lines contained in addition to the expected M-MuLV proteins a protein of 110,000 daltons immunoprecipitated by the goat anti-Moloney serum.

^e This cell line contained in addition to the A-MuLV P90 protein a novel protein of 85,000 daltons immunoprecipitated by both the goat anti-Moloney and anti-Abelson tumor sera.

as short as 5.0 kilobases and as long as 9.8 kilobases. In some cases (clones C1, C2, C3, C7, C8, C9, and C14), the *Hind*III site present at the 3' end of the A-MuLV DNA in the primary focus had been carried over into the secondary foci. Thus, the recombination which appended the helper sequences must have occurred to the right of the *Hind*III site in the primary provirus. In other cases (clones C4, C5, and C12), this site was lost, suggesting that the recombination occurred to its left. The length of the A-MuLV genome that was rescued was therefore variable. Similarly, in some cases many sites in the M-

MuLV genome were present in the restored 3' end, whereas in other cases only sites derived from the M-MuLV LTR were found. Thus, variable amounts of helper sequences had been appended. It is likely that many sites within both the A-MuLV parent and the M-MuLV helper can be utilized for recombination. The requirement for recombination between the helper virus and the truncated A-MuLV to give rise to the secondary foci presumably accounts for the low titer of focus-forming units released by the primary foci. Once this recombination has occurred, a fully transmissible virus is produced.

FIG. 5. Analysis of proviral DNA in cell lines infected with virus rescued from transfected cells. DNA was isolated, fractionated, and blotted as described in Fig. 1. Proviral DNAs were detected by hybridization with a labeled A-MuLV-specific probe. A: DNAs of the indicated cell lines were digested with *Xba*I (X), *Xba*I and *Hind*III (X + H), or *Hind*III (H) before electrophoresis. Each line contained the fragments expected from the endogenous *C-abl* gene (marked C for *Xba*I and *Hind*III digests) plus a new proviral band (marked V for the outer lanes). B: DNAs of the indicated cell lines were digested with *Sac*I. Each line contained the endogenous *C-abl* fragments (C) and one new proviral fragment of variable size. The provirus of line C5 comigrates with an endogenous fragment. C: DNAs were digested with *Kpn*I. Each line contains the expected endogenous fragments (C) and a new provirus. The provirus of line A14 comigrates with an endogenous fragment.

To examine the number of proviruses in secondary transformants, cleavage with *Hind*III was used. This enzyme released one to three new fragments containing A-MuLV sequences from the DNA of secondary foci (Fig. 5A). Because *Hind*III does not cleave in the LTR, each integrated provirus should give rise to one fragment labeled by the A-MuLV probe whose size will vary depending on the flanking cellular sequences. Because the number of proviruses seen will be a measure of the number of proviruses, from one to three proviruses are present in the cells of each secondary focus (Fig. 6). Because cleavage with enzymes which cut in the LTR gave only one size of DNA, the multiple proviruses in any given clone were apparently identical to one another. These multiple proviruses probably result from rescue of the initially infecting A-MuLV genome by the excess helper M-MuLV and then self-infection before the superinfection barrier appears (D. Steffen and R. Weinberg, unpublished data). There were two exceptions to this pattern (clones A9 and C10). In each of these lines, two different kinds of provirus were found.

A minority of the secondary foci (clones A14, C6, C8, C10, and C13) apparently contained A-MuLV DNA which had not simply recombined with helper sequences. Cleavage of DNA from

these cells by the three enzymes used did not yield positions consistent with the known location of these sites in the M-MuLV genome. The location of the 3' LTR is thus uncertain in these proviruses. Possibly, the A-MuLV has recombined with aberrant helper M-MuLV sequences or has suffered secondary rearrangements after recombination. Alternatively, helper sequences may not have been appended at the 3' end at all. The restriction sites mapped to date on these rare aberrant clones are shown in Fig. 7. It should be noted that two of these rare cell lines (clones A14 and C6) are rather low level producers of A-MuLV (Table 2). Thus, the A-MuLV provirus may have been transmitted to these secondary foci without complete repair of the 3' end. Some of these lines, however, do release high titers of A-MuLV and presumably have a functional (though aberrant) 3' end.

The size of the new A-MuLV genomes was also determined by an independent test. The secondary cell lines were metabolically labeled with $^{32}\text{P}_i$, and the labeled virions released into the medium were purified. The virion RNA was isolated and analyzed by electrophoresis through agarose gels after denaturation with glyoxal. Autoradiography (Fig. 8) revealed the presence of new RNAs of variable length, along with the expected M-MuLV RNA, 30S viral

FIG. 6. Structure of proviruses in each of two transfected cell lines (TFA-1 and TFC-1) and in each of 20 lines derived by infection with virus rescued from these two transfected lines. The position of the new restriction cleavage sites at the 3' end are shown. Because an *abl* probe was used as described in Fig. 5, only the cleavage sites nearest to the *abl* sequence were evident from the analysis. The *Xba*I, *Kpn*I, and *Sac*I sites mapping within the new 3' LTR have been left out for clarity. The position of the new 3' LTR (deduced from the position of these sites) is indicated by a box. The minimum extent of the new M-MuLV sequences (determined by the position of new, previously mapped M-MuLV restriction sites) is indicated by the shaded region. Note that the *Xba*I and *Kpn*I sites in C3 are different ones from those found in other clones but these sites are known to be present in the M-MuLV sequence. The number of identical proviruses in each cell line is indicated. The genome size in kilobases as deduced from the DNA structure, extending from positions in the left LTR to the equivalent position in the right LTR, is indicated. The viral RNA size, as measured directly on agarose gels (see Fig. 8), is indicated. N.D., Not determined.

FIG. 7. Structure of aberrant proviruses in each of six lines derived by infection with virus rescued from two transfected lines. The DNAs were analyzed as in Fig. 6. The restriction sites mapped are shown; no LTR can be placed on these maps. The sizes of the viral RNAs released by the cell lines are shown.

FIG. 8. Analysis of virion RNAs released by infected cell lines. After cells were metabolically labeled with $^{32}\text{P}_i$, virion RNA released into the medium was purified and analyzed by electrophoresis in agarose. Each cell line released particles containing a viral RNA of variable size along with the expected M-MuLV RNA, 30S virus-related RNA, and contaminating rRNA's.

RNAs (5, 15) and contaminating rRNA's. The size of the new RNA was in good agreement with the provirus DNA size contained in that cell line (Fig. 6). In some cases (clones A12, C1, C8, and C10), multiple new RNAs of several sizes are detected. The origin of these multiple RNAs is not known. Many of the cell lines containing aberrant proviruses (with unknown genome size) made multiple new RNAs.

The proteins produced by the secondary foci were also examined by immunoprecipitation with sera (anti-Abelson tumor and goat anti-Moloney) specific for A-MuLV and M-MuLV proteins (Fig. 9). All of the clones produced the expected M-MuLV proteins because they were producer cells. Most synthesized the A-MuLV-specific P90 protein as well (Table 2), although the level of protein detected was variable; in some clones (clones C8 and A12), the amount was very low. Several clones seemed also to produce a protein of approximately 110,000 daltons which was precipitated by the goat anti-Moloney serum and not by the anti-Abelson tumor sera (Fig. 9). The nature of this protein has not been determined.

High-efficiency transfection by A-MuLV DNA. Although a few foci could be recovered by direct transfection of cloned A-MuLV DNA, the efficiency of the process was extremely low. We therefore sought to modify the DNA structure to enhance the transfection frequency.

The A-MuLV insert present in λAb1 was

excised by cleavage with *HindIII* and ligated to *HindIII*-cleaved DNA of the plasmid vector pBR322 (7). The resulting DNA was introduced into competent bacterial cells, and ampicillin-resistant colonies were selected. Plasmid DNA from most of these clones showed a single copy of the A-MuLV insert in pBR322; these clones showed biological activity in animal cells similar to the original λAb1 clone (data not shown). One plasmid was recovered which consisted of a head-to-tail tandem dimer of Ab1 DNA inserted into a single pBR322. This plasmid, termed pAb1Ab1, thus contained a full size A-MuLV DNA genome.

Transfection of NIH/3T3 cells with this dimeric DNA was no more efficient than with the monomeric, permuted DNAs (Table 3A). Thus, reconstruction of the missing 3' terminus did not measurably increase the low transfection efficiency because only one clone (TFE-1) was recovered from several transfections, each using 100 ng of DNA. Analysis of the viral DNA in this clone suggested that a complete provirus, from 5' LTR to 3' LTR, had been introduced. Cleavage with all enzymes with sites in the LTRs released fragments whose sizes were indicative of a normal proviral structure (Fig. 2). Viral RNA was also detected in these cells (Fig. 3) some of which comigrated with authentic viral RNA. The cells of this focus were nonproducers. After superinfection with helper M-MuLV virus, the cells became high level producers of M-MuLV and, significantly, also high-level producers of A-MuLV (Table 2). Thus, A-MuLV DNA which was introduced into cells by trans-

FIG. 9. Analysis of viral proteins synthesized by infected cell lines. Proteins were immunoprecipitated and detected as in Fig. 4. M: goat anti-Moloney MuLV serum. A: mouse anti-Abelson tumor regressor serum. Each line contains the M-MuLV-specific proteins Pr65^{gag} and Pr80^{env} and the A-MuLV-specific P90. (Cell line C8 has very little P90.)

fection could be directly rescued at a high level by helper virus when a complete provirus was present. This result lends further support to the notion that repair of the 3' end missing in transfectants TFA-1 and TFC-1 is responsible for the low but significant level of A-MuLV rescuable from these lines.

Because modification of the cloned DNA did not appear to increase its transfectability we turned to cotransfection of the intact λ Ab1 DNA with a biologically active M-MuLV proviral clone termed pZAP (29). This yielded a considerable improvement in transformation efficiency. In one experiment, 100 ng of A-MuLV DNA yielded five foci (Table 3). Because helper virus was spreading throughout the culture, the transfectants were expected to be producers of M-MuLV; they might also have been producers of A-MuLV. Analysis of the virus harvested from individual foci (Table 2; TFB clones) showed that all were in fact high producers of both M-MuLV and A-MuLV. Analysis of the proviral A-MuLV DNA in these cultures (Fig. 10) showed that several proviruses were present in the DNA of each clone, and that an LTR had apparently been appended to the 3' end of each A-MuLV genome. At least some of the integrated DNAs had genomes of different lengths, suggesting that independent recombinational events had given rise to these genomes. The five clones, therefore, did not all arise by virus spread from a single producer cell. These transfectants were probably equivalent to the secondary foci derived earlier; helper virus had rescued the A-MuLV from rare transfectants, repaired the 3' end in one of several ways, and passed the genomes to the cells finally recovered as foci. Thus, transfection, repair, and rescue of A-MuLV can be combined in a single operation by the addition of helper M-MuLV DNA to the

transfecting A-MuLV DNA. The total efficiency of the process, however, was still low.

A very high efficiency of transfection was finally obtained by combining cotransfection with the use of rescuable A-MuLV cloned DNA. Dimeric A-MuLV DNA (such as pAb1Ab1), when mixed with helper M-MuLV DNA (such as pZAP), was capable of inducing transformation of cells at high efficiency (Table 3A). Typically, hundreds to thousands of foci were obtained with quantities of DNA (100 ng) which, without helper DNA, rarely gave even one focus. The helper DNA probably acted to pass the A-MuLV genome to new cells by infection, because cells which were previously infected with M-MuLV and therefore resistant to infection because of surface interference could not be transformed at this high level. Such cells, however, could be transformed by other selectable markers at control levels (Table 3A).

The large number of foci appeared very early after transfection (visible in less than 10 days) and were not localized near a large focus in the way that secondary foci normally surround a primary focus. The hundreds of foci thus did not seem to have spread from a rare transformant. To test this point more carefully, the A-MuLV was diluted and titrated in the presence of a constant amount of M-MuLV DNA (Table 3B). The number of foci observed showed a linear response to dilution of the A-MuLV DNA. Moreover, many foci were recovered with very small amounts of A-MuLV—far less than had given one focus in the absence of helper DNA. Thus, helper DNA did not merely spread A-MuLV from those rare cells which would have grown to foci; it rescued A-MuLV from many cells which otherwise would not have been stably transformed.

Two other full-length clones of the A-MuLV

TABLE 3. Number of foci produced by transfection of various forms of A-MuLV DNA

A. DNA	Helper DNA ^a	Recipient cells	Foci/100 ng
λ Ab1		NIH/3T3	~0.1
pAb1Ab1 (dimer)		NIH/3T3	~0.1
λ Ab1	pZAP	NIH/3T3	5
λ Ab1 cleaved and ligated	pZAP	NIH/3T3	42
pAb1Ab1 (dimer)	pZAP	NIH/3T3	600, 650
pAb1Ab1 (dimer)	pZAP	NIH/3T3/Mo ^b	0.5, 0.3
pSV2-gpt		NIH/3T3	150
pSV2-gpt		NIH/3T3/Mo	138
B. DNA	Amt (ng)	Helper DNA ^a	Foci
pAb1Ab1 (dimer)	300	pZAP	>1,000
pAb1Ab1 (dimer)	100	pZAP	594
pAb1Ab1 (dimer)	30	pZAP	191
pAb1Ab1 (dimer)	10	pZAP	65

^a A fixed amount (800 ng) of pZAP DNA (plasmid DNA containing a complete M-MuLV provirus) was used as helper where indicated.

^b NIH/3T3/Mo denotes NIH/3T3 cells chronically infected with Moloney murine leukemia virus.

FIG. 10. Analysis of A-MuLV proviruses in cells transfected with a mixture of permuted A-MuLV DNA and proviral M-MuLV DNA. DNAs were fractionated after cleavage with *Xba*I (X) or *Hind*III (H) and hybridized with an A-MuLV-specific probe. In addition to endogenous *C-abl* fragments (C), each line contains several proviruses (detected as new bands in the *Hind*III digests). Many lines contain proviruses of different sizes (as determined by bands in the *Xba*I digests).

genome (λ Ab3 and λ Ab4) which were previously isolated (9) were retested for biological activity under the conditions giving a high efficiency of transfection. No foci were observed. In addition, clones of a transformation-defective variant of A-MuLV (clones λ Ab2, λ Ab7, and λ Ab8) were similarly inactive.

Inhibition of gene transfer by A-MuLV DNA. The low frequency of direct transformation by the A-MuLV genome might be due to a low frequency of uptake, integration, or expression of the DNA. Alternatively, when the DNA is introduced by transfection, but not by infection, the DNA itself or the expression of gene products encoded by the DNA might be toxic to cells and prevent the outgrowth of a transformed focus. To examine these possibilities, the effect of A-MuLV DNA on transfer of a selectable gene was determined. A fixed amount of the selectable marker carried by a simian virus 40-*E. coli* xanthine-guanine phosphoribosyltransferase gene chimera (pSV2-gpt; reference 18) was mixed with test DNAs and applied to NIH/3T3 cells. Transformed colonies were detected by their resistance to mycophenolic acid (19). In one experiment (Table 4), 1 μ g of pSV2-gpt DNA alone gave rise to hundreds of colonies; addition of pBR322 DNA caused at most a slight reduction in the efficiency of stable transformation. Addition of an equivalent amount of A-MuLV DNA dramatically reduced the number of colonies detected. Thus, A-MuLV DNA or its gene products can apparently act in *trans* to

prevent stable transformation of cells by another selectable DNA.

DNA of a very similar but biologically inactive clone (λ Ab3) did not inhibit transformation of cells by pSV2-gpt. Moreover, cleavage of the biologically active clone (λ Ab1) to small fragments by *Pst*I also destroyed the inhibition activity of this clone (Table 4). Thus, the expression of A-MuLV transforming activity appears responsible for inhibition of transformation. This inhibition is apparently an effect of the biologically active gene products of the A-MuLV DNA and not of the DNA itself.

DISCUSSION

Transfection with A-MuLV DNA. Of three full-length clones of the A-MuLV genome previously isolated (9), one was found to be biologically active, whereas two apparently indistinguishable clones were not. These inactive clones probably harbor point mutations or other undetected alterations which affect the expression of the transforming gene. This mutation frequency (2/3) is in agreement with the high levels of mutation noted for retrovirus replication (10, 28) and is also comparable to that seen in clones of M-MuLV (unpublished observations) and of another strain of A-MuLV encoding the larger P160 transforming protein (S. Latt, personal communication).

The insert in the active clone λ Ab1 was permuted relative to the normal integrated provirus of A-MuLV because it was prepared by *Hind*III cleavage of a circular replication intermediate. *Hind*III cleaves 3' to the region encoding the P90 transforming protein (11) and thus the LTR region (which carries the promoter for transcriptional initiation), the *gag* region, and the coding portion of the *abl* region were all retained in their normal order in the permuted clones. This presumably allowed the cloned DNA to retain its ability to transform cells.

The active clone also contained two tandem copies of the LTR because it was derived from a circular intermediate containing two LTRs (9). It is not known whether the two LTRs are necessary or helpful for biological activity. In other experiments with M-MuLV clones (unpublished data), we have found no difference between the activity of clones carrying one or two LTRs.

Direct transfection with the permuted A-MuLV DNA yielded a few foci which carried A-MuLV DNA. The viral DNA in these cells was incomplete; analysis of the restriction sites surrounding the viral DNA suggested that the 3' end of the genome was missing. Thus, the 3' untranslated end of A-MuLV was apparently not required for morphological transformation. In fact, restoration of the 3' end to the transfecting

TABLE 4. Inhibition of transformation by A-MuLV DNAs

Expt no.	Selected DNA ^a	Mixed with ^b :	Colonies/100 ng of selected DNA
1			0
	pSV2-gpt		94
	pSV2-gpt	pAb1Ab1	5
	pSV2-gpt	pBR322	54
2			0
	pSV2-gpt		47, 55
	pSV2-gpt	pAb1Ab1	8, 4
	pSV2-gpt	pAb1Ab1 cleaved by <i>Pst</i> I	55, 54
	pSV2-gpt	λ Ab3	38, 48
	pSV2-gpt	pBR322	61, 43

^a A fixed amount (100 ng) of the selectable DNA was used.

^b One microgram of the cloned A-MuLV was used; an equivalent amount on a molar basis of pBR322 DNA (260 ng) was used. All points contained 75 μ g of carrier DNA.

DNA by dimerization of the insert (Table 3) did not improve the transfection efficiency. Apparently, sequences which are randomly appended to the 3' end of the incomplete provirus during the transfection process do not block expression of the virus, because large amounts of virus-specific RNA and virus-encoded P90 protein were detected in these cells. It is not clear whether any special sequences were selected by the need for expression of the partial A-MuLV genome.

Only one or two distinct DNA fragments containing the A-MuLV DNA were found in the primary foci; the high intensity of hybridization suggested that these fragments were present at greater than a single copy per haploid genome. Thus, as has been observed in other systems (12), the transfected DNA was apparently amplified in such a way as to preserve the flanking sequences. The very high copy numbers (greater than 100) occasionally found after transfection with ϕ X DNA and the thymidine kinase gene (33) were not observed with A-MuLV DNA.

In at least two cases, the lambda phage vector DNA was also carried into the cell and became stably associated with the cellular DNA (Fig. 1). This was found whether or not the A-MuLV insert was excised from the vector DNA. However, the lambda DNA in these cells was never so closely linked to the A-MuLV as to be joined on the same restriction fragment. The lambda DNA did not stay joined even when the DNA applied to the cells was uncleaved (focus TFA-1, Fig. 1). Thus, in our cells, the transfecting DNA is apparently fragmented and subsequently joined to new DNAs at random before becoming stably associated with the cell DNA.

Rescue of defective proviruses. A-MuLV partial genomes could be rescued from the primary foci by helper virus. In most cases, it could be

demonstrated that rescue was accompanied by restoration of the 3' end of the genomes; M-MuLV DNA (at least three different restriction sites in the DNA) was appended to a portion of the A-MuLV genome. Apparently new sequences were appended by recombination with the helper M-MuLV, as was first shown by Goldfarb and Weinberg (13) for the rescue of partial proviruses of Harvey sarcoma virus. The new A-MuLV genomes created by the recombination had various sizes, ranging from 5.0 to 9.8 kilobases in length. In addition, the amounts of A-MuLV and of M-MuLV present in the recombinant were variable. In some cases, all of the available A-MuLV DNA and at least some of the 3' flanking sequences were probably incorporated into the recombinant; in other cases, not even all of the permuted A-MuLV genome was present. Once recombination had occurred, the new genomes were capable of normal high-efficiency transfer to new cells mediated by helper virus. Several of the new genomes were extremely large, even larger than the helper RNA, and were nevertheless capable of being packaged into virions and transmitted to new cells. These observations extend the known upper limit for packaging by murine retrovirus virions to nearly 10 kilobases. Some of these larger genomes contain a complete *env* gene and thus might be capable of helper-independent expression of Pr80^{env}. Experiments are in progress to analyze further the proteins encoded by these large viruses.

All of these viruses encode the A-MuLV-specific P90 protein rather than the full-length P120 protein because they were derived from a strain encoding the shortened protein P90 (9); the P90A strains contain a point mutation or other small alteration causing early translational termination (11). The sequences immediately

following the termination site for P90 are unchanged and thus code for the extra 30,000 daltons of protein present in P120 and missing in P90. In the clone containing the least A-MuLV DNA (clone C4), the amount of the *abl* region present was barely able to encode the *abl*-specific portion of the wild-type P120 protein. We can be certain, therefore, that the downstream sequences (after the C-terminus of the P120) are not needed for transformation of fibroblasts. Apparently, expression of P90 is sufficient.

Several rescued viruses contained new sequences at the 3' end, but the restriction sites seen did not suggest the addition of normal helper sequences as was found in most of the cases. Many explanations for these unusual proviruses are possible. One possibility is that the helper virus parent of these recombinants may have been a variant with an unusual 3' end (lacking one of the sites expected). Sites would then be detected which do not all fall into the pattern seen with a normal helper virus.

It is also possible that no LTR is in fact present at the 3' end of these variant proviruses. Rescue and subsequent integration of the A-MuLV genome would have had to occur by an aberrant process not yielding a normal proviral structure. Rescue from primary foci was such a rare event, however, that very infrequent processes (such as aberrant integrations) could account for the observed frequency. This possibility is strengthened by the finding that at least some of these aberrant clones were not high-level producers of A-MuLV (Table 2). Thus, an independent functional test suggests that at least part of the structure needed for normal transmissibility was not present. At some low frequency, virus infection apparently resulted in integration of aberrant proviruses.

These aberrant secondary proviruses were not found in the initial discovery of the rescue process described by Goldfarb and Weinberg (13). Only four independent isolates were analyzed in that work and thus statistically it is possible that no aberrant proviruses were detected. However, proviruses in secondary foci were not directly analyzed; rather, nonproducer transformed cell lines (tertiary lines) were derived by infection with the virus released from the secondary foci (13). The RNA in these nonproducer lines was studied. Thus, if aberrant secondary proviruses were formed in that work they might have been repaired during transfer to the tertiary cells.

Further analysis of the RNA produced by these cells and of the proviruses in tertiary foci should determine whether LTRs are present and if so, whether they are transmitted without change to new cells during subsequent passages.

Efficiency of transfection. The direct transfection of A-MuLV DNA into NIH/3T3 cells occurs with very low efficiency compared with that of other selectable DNAs (Table 1). A few foci were recovered, but large numbers of cells and large amounts of DNA were required. The aberrant permuted structure of the DNA did not seem to be responsible for the low efficiency, since dimerization which reconstructed a complete provirus did not improve the yield. Vector sequences were also not responsible. Cotransfection of the dimeric A-MuLV DNA with proviral helper M-MuLV DNA, however, resulted in an enormous stimulation in the number of foci recovered. Apparently virus could be rescued from cells which would not otherwise have been stably transformed and transmitted as pseudotyped genomes to new cells. The process could occur without the 3' end of the A-MuLV genome but was carried out most efficiently when complete A-MuLV DNA was used.

We favor the hypothesis that a large number of A-MuLV genomes is typically introduced into recipient cells by DNA transfection. This large number may be due to the direct introduction of many copies or to amplification of the introduced DNA once it has entered the cell via transfection. We speculate as well that the multiple copies of A-MuLV are toxic to the cell and that most cells transfected with Ab1 DNA are inviable. Thus, the consequences of most successful gene transfers are obscured by the death of the transfected cells. Transfection is much more efficiently registered if the acquired A-MuLV is rescued from the transfected cell before it dies and is introduced by helper virus into neighboring cells. These neighboring cells acquire low numbers of A-MuLV by infection and are therefore able to grow out into transformed colonies. Such a mechanism explains as well the inhibitory effects of A-MuLV on the cotransfected pSV2-gpt cloned DNA. We suggest that both cotransfected DNAs become amplified in a recipient cell and that this cell is killed by the multiple A-MuLV genomes.

It is likely that the toxic effect is mediated by expression of the P90 protein and is not a direct effect of the A-MuLV DNA because inactive clones and fragmented clones (Table 4) did not show the effect. The presence of large amounts of P90 and its associated kinase activity may cause a drastic disruption of cellular metabolic control.

The transfection of total cellular DNA isolated from A-MuLV-transformed cells has been recently described (16). In these experiments, the transfected cells were passaged many times before a final plating and scoring for the appearance of foci. Thus, the efficiency of the actual transfection procedure could not be determined.

The number of foci seen, however, is not inconsistent with our results. Recently a cloned provirus copy of the A-MuLV genome was used by others to transform NIH/3T3 cells (31) and was found to show high-efficiency transformation even without helper virus. We have obtained this clone and tested the efficiency of transformation under our conditions. Both the low efficiency and the stimulation upon addition of M-MuLV DNA were identical to those obtained with our cloned DNA. Thus the recipient cells or the conditions of transfection must be responsible for the difference in results between the laboratories.

ACKNOWLEDGMENTS

We thank Ann Gifford for excellent technical assistance.

This work was supported by Public Health Service grant CA-17537 (to R.W.), program project grant CA-26717 (to D.B.), and grant CA-14051 (core grant to S. E. Luria), all from the National Cancer Institute. J.Y.-J.W. is a postdoctoral fellow of the Jane Coffin Childs Memorial Fund for Medical Research. D.B. is an American Cancer Society Research Professor.

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