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Isolation of Recombinant DNA Clones Carrying Complete Integrated Proviruses of Moloney Murine Leukemia Virus

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*Eco*RI DNA fragments from a Moloney murine leukemia virus (M-MuLV)-infected mouse fibroblast line (M-MuLV clone A9) were cloned in lambda phage Charon 4A cloning vector to derive clones containing integrated M-MuLV proviral DNA. A 10- to 16-megadalton class of *Eco*RI fragments was chosen for cloning, based on (i) its ability to induce XC-positive virus upon transfection of NIH/3T3 cells, and (ii) its content of a 0.8-megadalton viral *Kpn*I fragment diagnostic for M-MuLV. Six recombinant DNA clones were isolated which contain a complete M-MuLV provirus, as judged by (i) restriction endonuclease mapping and (ii) the fact that all of the clones gave rise to XC-positive, NB-tropic virus upon DNA infection in NIH/3T3 cells. The sizes of the inserts were 12.0 (for three clones) or 12.5 megadaltons (for three clones). Restriction mapping indicated that these six clones represent five different M-MuLV proviral integrations into different cellular DNA sites.

Moloney murine leukemia virus (M-MuLV), like other murine and avian retroviruses, establishes a stable association with the host cell that it infects. During infection, one or more DNA copies of the viral RNA genome are covalently integrated into the chromosomal DNA of the host cell to form proviruses (26). In both avian and murine systems, multiple sites for integration of retrovirus DNA sequences into cellular DNA have been demonstrated by restriction enzyme mapping (1, 8, 14, 16, 24). Although it seems clear that integration is not limited to a single or a very small number of chromosomal locations, it is as yet unclear whether integration into cellular DNA sequences occurs totally at random or whether there are regional and/or sequence preferences for M-MuLV integration.

Although multiple integrations of M-MuLV and other retroviruses can occur in a single cell, not all such integrations are functionally equivalent. Keshet and Temin (3, 18) studied integrations of reticuloendotheliosis virus and spleen necrosis virus DNAs. They demonstrated a wide size range of provirus-containing restriction enzyme fragments in both acute and chronically infected cells. Viral infectivity, as measured in a transfection assay, however, resided in a single restriction enzyme fragment size (3, 4). Jaenisch has derived a series of mouse strains which genetically transmit M-MuLV DNA sequences inserted at different chromosomal locations (17, 17a). The patterns of viral gene expression, as well as the effects on mouse development, vary among the different proviral integrations and provide strong evidence for "position effects," or regulation of the expression of the inserted M-MuLV DNA.

We (7) have indirectly studied the transcriptional activity of integrated M-MuLV genomes in a number of infected fibroblast lines by measuring the relative DNase I sensitivity of M-MuLV DNA sequences in isolated nuclei. Weintraub and Groudine (29) have shown that the DNA of actively transcribed genes is preferentially digested when nuclei or chromatin is treated with pancreatic DNase I. In cells with several integrated copies of M-MuLV, only a minority (as few as one copy) of the viral DNA sequences were found in a preferentially DNase I-sensitive chromatin configuration. Most proviral copies were resistant, suggesting that many of the integrated M-MuLV copies in these cells were not actively transcribed. Thus, fibroblasts, productively infected with M-MuLV, apparently contain both transcribed and nontranscribed copies of proviral DNA.

Further studies of the fine structure and expression of integrated M-MuLV genomes would be greatly facilitated by the availability of recombinant DNA clones which contain integrated M-MuLV proviral DNA and the surrounding cellular DNA sequences. We report here the isolation and preliminary characterization of a series of lambda phage recombinant DNA clones which contain such integrated M-MuLV proviral DNAs from an infected mouse fibroblast line.

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MATERIALS AND METHODS

All cells were maintained in Dulbecco-modified Eagle medium supplemented with 10% calf serum (GIBCO Laboratories, Grand Island, N.Y.). The derivation of M-MuLV clone no. 1 (10) and M-MuLV clone A9 (9) has been previously described.

Isolation of high-molecular-weight DNA and unintegrated M-MuLV viral DNA, the synthesis of high-specific activity ^{32}P-labeled M-MuLV representative complementary DNA (cDNA), and blot-transfer hybridization (23) have been previously described (2, 11).

Restriction enzyme digestions, gel electrophoresis in horizontal agarose gels, and blot transfer to nitrocellulose were performed as previously described (2). For preparative isolation of DNA fragments, DNA samples were electrophoresed into thick (2-cm) 0.6% agarose gels, the DNA was visualized under UV light after staining with ethidium bromide, and appropriate regions of the gel were excised. The gel was dissolved by heating in 10 volumes of 8 M $NaClO_4$ at 60°C for 1 to 2 h and then passed over a column of hydroxyapatite (BioRad), equilibrated in 0.05 M PB (sodium phosphate buffer, pH 6.8) at 60°C. The column was washed with 10 column volumes of 1 M $NaClO_4$–0.05 M PB, and bound DNA was eluted with 0.5 M PB. The eluted DNA was dialyzed against 0.1 M ammonium acetate and concentrated by lyophilization.

Ligation. A 1-μg amount of gel-purified cellular DNA was mixed with 2 μg of an equimolar mixture of Charon 4A lambda phage left and right end fragments and lyophilized. The DNA was resuspended in 0.01 M Tris (pH 7.4)–0.01 M $MgCl_2$–0.05 M NaCl at a concentration of 300 μg/ml and incubated at 42°C for 1 h. The solution was adjusted to 0.4 mM ATP and 1 mM DTT, 2 U of T4 DNA ligase (Bethesda Research Laboratories, Bethesda, Md.) was added, and incubation was continued for 16 to 48 h at 12°C.

In vitro packaging. Products of the ligation reactions were packaged into lambda phage particles with purified lambda protein A, freeze-thaw lysates, and sonic extracts of induced lambda lysogens by the protocol of Blattner and co-workers (25; H. Faber, D. Kiefer and F. Blattner, personal communication, protocols distributed with Charon vectors, 1978). In one 600-μl packaging reaction, 2.1 μg of ligated DNA resulted in 2.2×10^4 recombinant phage. In a second 600-μl packaging reaction, 7.2×10^5 recombinant phage resulted from the same amount of input DNA. The differences in efficiencies may have been due to different extents of ligation in the two DNA samples.

All packaging experiments and subsequent biological manipulations were performed in a certified P-2 containment laboratory.

Screening of recombinant DNA clones. Products of the in vitro packaging reactions were adsorbed to *Escherichia coli* DP50 *supF* and plated onto 12 15-cm petri dishes containing NZYDT agar (6). Nitrocellulose replicas of the petri dishes were prepared by the method of Benton and Davis (5) and hybridized with ^{32}P-labeled cDNA. Dextran sulfate (10%) was added to the hybridizations to accelerate the annealing rate (28). After autoradiography of the nitrocellulose replicas, plaques on the agar plates which showed hybridization with the M-MuLV cDNA probe were picked and subjected to additional cycles of plaque purification and screening until more than 90% of the plaques reacted positively with the M-MuLV cDNA probe. A final plaque was then picked and used to prepare a high-titer liquid lysate.

In one experiment, approximately 5×10^3 recombinant phage were screened, and 12 plaques hybridized M-MuLV cDNA. Of these 12, 8 hybridized strongly and 1 was subsequently found to contain a M-MuLV proviral integration. In a second experiment, approximately 1.5×10^4 recombinant phage were screened, and 30 plaques hybridized M-MuLV cDNA, of which 11 strongly hybridized. Six of the strongly hybridizing clones were further analyzed, and five contained M-MuLV provirus whereas the other contained MuLV-related endogenous virus sequences.

Preparation of recombinant phage. Bulk preparation of recombinant phase was performed by the protocol of Blattner (Blattner et al., charon phage protocols, 1978). Briefly, high-titer lysates were used to infect *E. coli* DP50 *supF*, and mass liquid cultures were grown. After lysis, the supernatant was clarified and phage were precipitated by addition of polyethylene glycol. Precipitated phage were then banded through a step CsCl gradient followed by banding to equilibrium in CsCl. In cases where multiple phage bands resulted (see Discussion), the individual bands were harvested separately.

DNA infections. NIH/3T3 cells were infected with 0.5 to 2.0 μg of purified recombinant phage DNA per 5-cm dish 1 day after seeding NIH/3T3 cells at 5×10^5 cells. Infections were carried out by a modification of the calcium phosphate precipitation technique of Graham and van der Eb (15) by using calf thymus DNA carrier and dimethylsulfoxide treatment as described by Lai and Verma (19). Cultures were assayed for virus-infected cells by UV-XC plaque assay (22) or passaged several times to establish a chronically infected culture.

RESULTS

The source of infected cell DNA for these molecular cloning experiments was the cell line M-MuLV clone A9. M-MuLV clone A9 cells are a clone of NIH/3T3 fibroblasts productively infected with M-MuLV, and we have previously characterized them as containing approximately 4 copies of M-MuLV DNA per haploid genome, or 10 to 12 copies per cell (7, 9). *Eco*RI DNA fragments containing some of these integrated M-MuLV proviral DNAs (approximately 13.0, 12.0, 8.7, 5.4, and 4.6 megadaltons [Md]) have been identified in these cells (1, 2). In addition, even though this cell line is productively infected, only a minority of the viral DNA sequences exist in chromatin in a configuration which is preferentially sensitive to DNase I digestion and therefore potentially transcriptionally active (7).

We wished to clone M-MuLV proviruses from an infected mouse cell, the natural host for M-MuLV, and from a characterized cell clone to

facilitate further studies of the structure and function of individual M-MuLV integrations. A serious problem in attempting to screen for M-MuLV proviral DNA sequences in mouse cells is the presence of an endogenous family of MuLV-related sequences which share partial sequence homology with M-MuLV. Figure 1A shows the complex pattern of *Eco*RI fragments with nucleic acid sequence homology to a representative M-MuLV cDNA probe (11) from uninfected mouse cells and M-MuLV clone A9 cells. Two *Eco*RI fragments present only in the infected cell line and absent from the uninfected cells can be seen at approximately 12×10^6 to 13×10^6 daltons, and they may contain integrated M-MuLV proviral DNA. That these new *Eco*RI fragments actually contain integrated M-MuLV DNA sequences is shown by their enhanced hybridization to an M-MuLV cDNA specific for M-MuLV DNA (and which does not recognize endogenous MuLV-related sequences in uninfected cells) as shown in Fig. 1B (2).

Our experimental design involved cloning of size-selected *Eco*RI fragments from total M-MuLV clone A9 DNA in the lambda phage charon 4A cloning vector (6). This strategy was employed for several technical reasons. First, *Eco*RI does not cleave M-MuLV DNA (12), so *Eco*RI fragments from infected cell DNA would contain complete copies of MuLV proviral DNA with adjacent 5′ and 3′ cellular sequences. Second, the Charon 4A cloning vector was designed for cloning of *Eco*RI fragments, and the sizes of inserted DNA fragments that can be accommodated by this vector (7.6 to 20.6 kilobase pairs or 4.85 to 13.2 Md) include most of the *Eco*RI fragments containing M-MuLV proviral DNA in M-MuLV clone A9 cells. Third, size selection of the *Eco*RI fragments was deemed necessary due to the large number of *Eco*RI fragments containing endogenous M-MuLV-related sequences present in both uninfected and infected cells. Generating lambda phage clones from total infected cell DNA would result in a large number of clones containing endogenous MuLV-related sequences in addition to the smaller number of desired M-MuLV proviral clones. Clones of these endogenous virus-related sequences would also be recognized during the screening procedures. Thus, many clones which hybridize M-MuLV cDNA might have to be screened before one containing a genuine M-MuLV provirus might be obtained. As shown in Fig. 1A, certain sizes of *Eco*RI fragments from M-MuLV clone A9 cells contain relatively high concentrations of M-MuLV proviral DNA in comparison to endogenous M-MuLV-related sequences, for instance, the 12×10^6- to 13×10^6-dalton range.

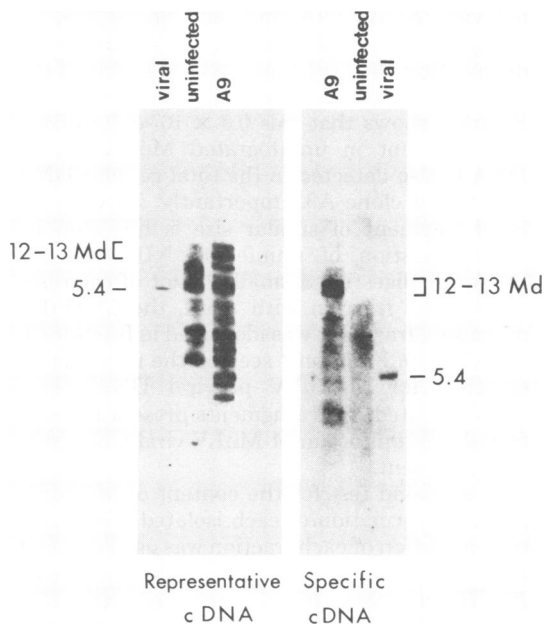

FIG. 1. *Identification of M-MuLV proviral fragments. Uninfected NIH/3T3 and infected M-MuLV clone A9 cell DNAs (10 μg per channel) were digested with EcoRI and analyzed by agarose gel electrophoresis and blot transfer to nitrocellulose filters. As a mobility marker, linear unintegrated M-MuLV DNA (5.4 Md) isolated from freshly infected cells was also included ("viral"). Hybridization to two replicate blots was performed with either representative M-MuLV cDNA ("representative cDNA") or with cDNA specific for M-MuLV which was generated by preannealing representative M-MuLV cDNA with a large excess of AKR MuLV 70S RNA ("specific cDNA"). Autoradiograms of the hybridized blots are shown.*

Therefore, appropriate size selection of the M-MuLV clone A9 *Eco*RI fragments before cloning could provide an important enrichment of M-MuLV proviral sequences over endogenous MuLV-related sequences. For these reasons. we chose to clone M-MuLV clone A9 *Eco*RI fragments of 12×10^6 to 13×10^6 daltons.

To prepare size-selected fragments, 10 mg of M-MuLV clone A9 cell DNA was digested with *Eco*RI and fractionated by electrophoresis in 0.6% agarose gels. DNA was extracted from slices of the agarose gel by dissolving the gel in concentrated $NaClO_4$ and binding the DNA to hydroxyapatite. Figure 2 shows the size distribution of each of these isolated fractions, which are also listed in Table 1. Each size fraction of infected cell DNA was tested for its content of integrated M-MuLV sequences by two additional criteria. First, a portion of each DNA fraction was tested for the presence of an inter-

nal viral *Kpn*I DNA fragment whose size, although not necessarily sequence composition, is diagnostic of M-MuLV as opposed to any of the murine endogenous virus-related sequences (2). Figure 3 shows that this 0.8 × 10^6-dalton fragment present in unintegrated Moloney viral DNA is also detected in the total cellular DNA of infected clone A9. Importantly, no virus-related fragment of similar size is liberated by *Kpn*I digestion of uninfected NIH/3T3 cell DNA. By digesting a small aliquot of each isolated DNA fraction with *Kpn*I, the M-MuLV diagnostic fragment was identified in fractions 4, to 7 (Fig. 3). Fraction 4 seemed the most highly enriched for M-MuLV proviral DNA, since MuLV-related *Kpn*I fragments present in uninfected cells but not in M-MuLV viral DNA were largely absent.

In a second test for the content of M-MuLV genetic information of each isolated DNA fraction, a portion of each fraction was used to infect NIH/3T3 cells in a DNA transfection assay. *Eco*RI-cut DNA from uninfected NIH/3T3 cells, total M-MuLV clone A9 DNA, and unintegrated viral DNA isolated from the cytoplasm of freshly infected cells were also tested. After one or more passages, each transfected culture was assayed for M-MuLV infection by an XC syncitial assay (22). Unintegrated viral DNA and total M-MuLV clone A9 DNA both produced XC-positive virus in the transfected cultures, whereas uninfected NIH/3T3 cell DNA did not. Fractions 4 and 5 also induced XC-positive virus, confirming their content of genet-

TABLE 1. *Characterization of size-selected EcoRI-cut DNA of M-MuLV infected cell line A9*

DNA fraction	Size range (×10^6 daltons)	M-MuLV-diagnostic *Kpn*I fragment	Infectivity
Unintegrated viral DNA	5.8	+	+
*Eco*RI-cut 3T3 DNA	<1->20	–	–
*Eco*RI-cut A9 DNA	<1->20	+	+
A9 fraction			
1	9–20	–	–
2	9–20	–	–
3	13.5–18	–	–
4	10.5–16	+	+
5	9.4–12	+	+
6	7–10	+	–
7	6.5–9	+	–
8	5.8–6.7	–	–
9	5.0–6.0	–	–
10	4.3–5.5	–	–
11	4.1–4.7	–	–

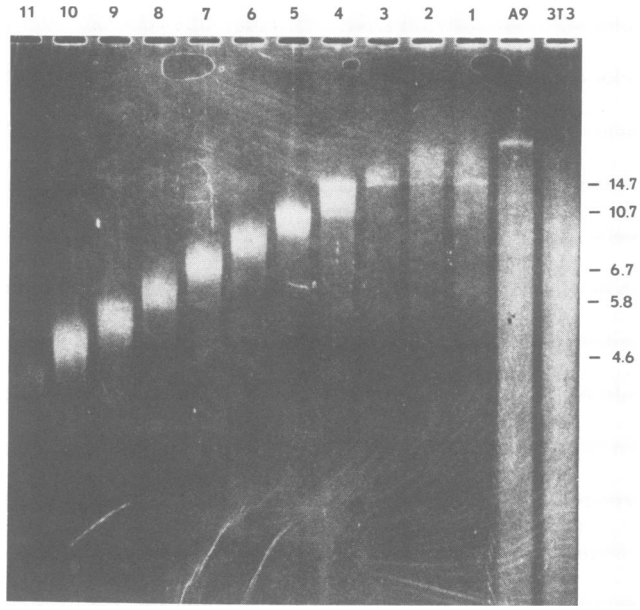

FIG. 2. *Characterization of size-selected DNA fractions. Aliquots of each size fraction (1 to 11) of purified EcoRI-cut M-MuLV clone A9 cell DNA were electrophoresed through a 0.6% agarose gel and visualized by staining with ethidium bromide. Also shown are total EcoRI digests of M-MuLV clone A9 cell DNA and uninfected 3T3 cell DNA. The positions of several restriction enzyme fragments of λ DNA analyzed on the same gel are indicated, along with their sizes in Md. λ DNA cut with EcoRI was mixed with the digested M-MuLV clone A9 DNA before preparative fractionation to provide guides for slicing the gel. These λ restriction enzyme fragments are visable in several isolated DNA fractions.*

ically competent M-MuLV (Table 1). It is interesting that fraction 7 may have contained M-MuLV proviral DNA by the *Kpn*I cleavage test, but did not yield infectious virus upon transfection.

DNA fraction 4, with a size range of 10.5×10^6 to 16.0×10^6 daltons, was cloned into the charon 4A cloning vector. The right and left EcoRI end fragments of charon 4A, purified by preparative agarose gel electrophoresis, were ligated with fraction 4 DNA, and the high-molecular-weight DNA was packaged into lambda particles in vitro by using the in vitro packaging system described by Blattner (see Materials and Methods). In vitro-assembled phage particles were directly plated as primary plaques on *E. coli* DP50 *supF* without initial amplification. Recombinant phage plaques were screened for sequence homology to M-MuLV by hybridization of an M-MuLV cDNA probe to nitrocellulose filter replicas of the initial plates (5). Plaques with strong sequence homology to M-MuLV were picked, and plaques were purified.

Recombinant DNA phage with strong sequence homology to M-MuLV could represent clones of intact integrated M-MuLV proviruses, rearranged or otherwise aberrant integrations of all or part of the M-MuLV genome, or clones of endogenous MuLV related sequences which reside on *Eco*RI fragments of the size selected for cloning. To distinguish between these possibilities, DNA was purified from each recombinant clone and further characterized with respect to its restriction enzyme cleavage pattern. Each DNA sample was digested with *Kpn*I to test for the presence of a M-MuLV-diagnostic 0.8×10^6-dalton fragment which is liberated from the 3′ region of the viral genome. Figure 4 shows the patterns of restriction enzyme fragments obtained from eight recombinant clones. Blot

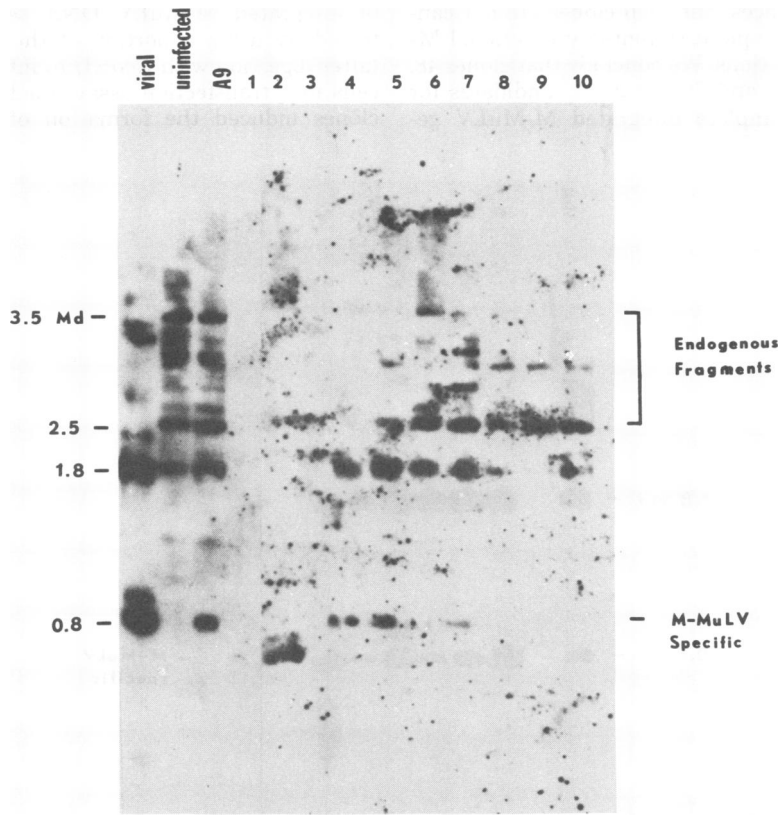

FIG. 3. *KpnI cleavage of size-selected DNAs. Aliquots of the size-selected EcoRI fragments from M-MuLV clone A9 cells were secondarily cleaved with KpnI and analyzed by electrophoresis and blot transfer hybridization by using a representative M-MuLV cDNA probe. For comparison, total cellular DNA from M-MuLV clone A9 and uninfected NIH/3T3 cells, as well as unintegrated M-MuLV linear DNA, was also digested with KpnI and analyzed. The mobility of the M-MuLV-diagnostic 0.8-Md KpnI fragment, as well as an endogenous 2.5-Md KpnI MuLV-related fragment present in uninfected cells but not contained in genuine M-MuLV provirus, is indicated.*

transfer hybridization of this gel with M-MuLV cDNA is shown. Six of the eight clones tested contained the M-MuLV diagnostic fragment of 0.8 Md. This suggests that they contain at least the 3′ portion of an M-MuLV genome, since this fragment is actually a doublet of two internal viral DNA fragments liberated from the 3′ end of the M-MuLV DNA sequences. These six clones also liberate a doublet of M-MuLV-hybridizing *Kpn*I fragments of 1.8 Md which arise from the 5′ end of M-MuLV. This fragment is not diagnostic of M-MuLV, however, since the vast majority of the MuLV-related endogenous sequences also contain a 1.8-Md *Kpn*I fragment (Dolberg, Bacheler and Fan, submitted for publication). Indeed, clone 27 contains such a virus-related 1.8-Md fragment, but lacks the diagnostic 0.8-Md fragment. It is possible that clones which fail to release the M-MuLV-diagnostic 0.8-Md *Kpn*I fragment carry M-MuLV sequences, either as partial integrations or rearranged sequences, but such clones are not candidates for complete, potentially functional M-MuLV integrations. We conclude that clones 48, 61, 63, 73, 75, and 76 are good candidates for containing complete integrated M-MuLV genomes. These six clones also contain a 1.9-Md M-MuLV-diagnostic fragment liberated by *Bam* digestion (1) which comigrated with the 1.9-Md *Bam* fragment from unintegrated M-MuLV viral DNA and which hybridized to M-MuLV cDNA (data not shown).

Figure 5 shows a determination of the sizes of the inserted cellular DNA sequences. DNA from each clone was cut with *Eco*RI which separates the vector sequences from the inserted DNA and analyzed by gel electrophoresis. The six clones identified as containing M-MuLV-diagnostic fragments fall into two size classes, having inserts of approximately 12.0 and 12.5 Md. The sizes are very similar to the 12 and 13 Md of the *Eco*RI fragments previously identified in total M-MuLV clone A9 DNA as containing integrated M-MuLV DNA sequences. The sizes of the inserts suggested that at least two different integrations of M-MuLV DNA were cloned.

The biological activity of each of the six clones of integrated M-MuLV DNA sequences was tested by using a portion of the phage DNA (after digestion with *Eco*RI) to infect NIH/3T3 cells in a transfection assay. Each of these six clones induced the formation of XC-positive

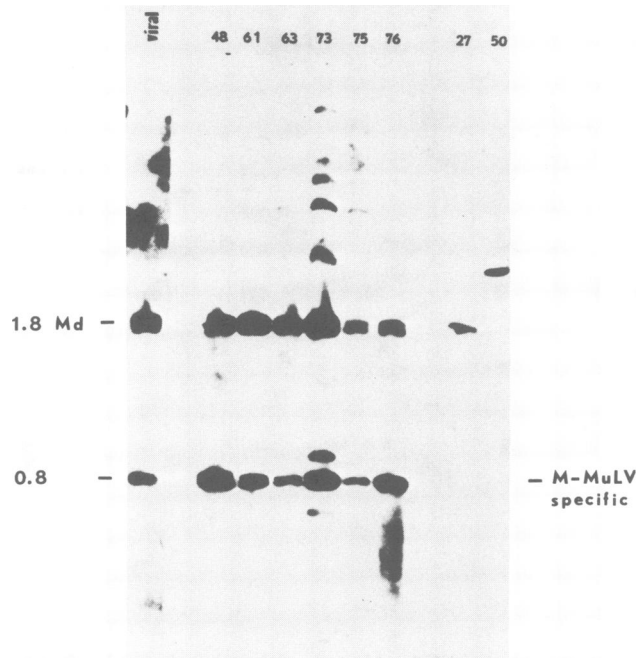

FIG. 4. *Test of recombinant clones for M-MuLV provirus. DNA from isolated recombinant clones or from linear unintegrated M-MuLV viral DNA purified from freshly infected cells ("viral") was digested with KpnI and electrophoresed through a 1% agarose gel. Hybridization to a blot transfer of this gel with M-MuLV-representative cDNA is shown. The mobility of the M-MuLV-diagnostic 0.8-Md KpnI fragment is indicated. The full designations of the clones are Ch4A-A9-4- followed by the clone number, but for simplicity are referred to by the clone number. In the figure, Ch4A-A9-4-48 is indicated as clone 48.*

plaques in the infected cell cultures, whereas other clones isolated in the same experiment, which did not contain M-MuLV diagnostic fragments, did not (clones 27 and 28) (Table 2). Supernatant virus from each XC-positive-infected culture was further characterized by titration on NIH/3T3 and BALB/c 3T3 cells. The XC-positive virus in each case titered with equal efficiency on both cell types, indicating that the viruses liberated after cloned DNA infection had the NB tropic host range of M-MuLV itself. This NB tropic host range is also not characteristic of any known endogenous murine virus. This further supports the identification of these recombinant DNA clones as containing intact integrations of M-MuLV DNA.

The two sizes of cellular DNA inserts containing integrated M-MuLV genomes suggested that at least two different integrations of M-MuLV DNA into cellular DNA sequences were cloned. To further characterize the inserted sequences, restriction enzyme maps of the six clones containing integrated M-MuLV proviral DNA were constructed by sequential restriction enzyme digestions and by blot transfer-filter hybridizations with M-MuLV cDNA. Figure 6 shows restriction enzyme maps for the *Eco*RI inserts of these six clones. The maps have been drawn to align the 5′ end of the integrated viral DNA sequences and indicate that the integrated viral DNA sequences are located in different positions in several of the clones. One 12.0-Md cellular DNA fragment was cloned twice, on clones 61 and 76, in opposite orientations with respect to the Charon 4A cloning vector. Restriction enzyme sites in the flanking cellular DNA sequences indicate that clone 73 contains a different M-MuLV integration than that contained in

TABLE 2. *Characterization of isolated M-MuLV-related recombinant DNA clones*

Clone no.	Size of insert ($\times 10^6$ daltons)	M-MuLV-diagnostic fragments	Infectivity	NB tropism
48	12.5	+	+	NB
61	12.0	+	+	NB
63	12.5	+	+	NB
73	12.0	+	+	NB
75	12.5	+	+	NB
76	12.0	+	+	NB
70	7.7	−	ND[a]	−
27	9.2	−	−	−
28	8.9	−	−	−

[a] ND, Not determined.

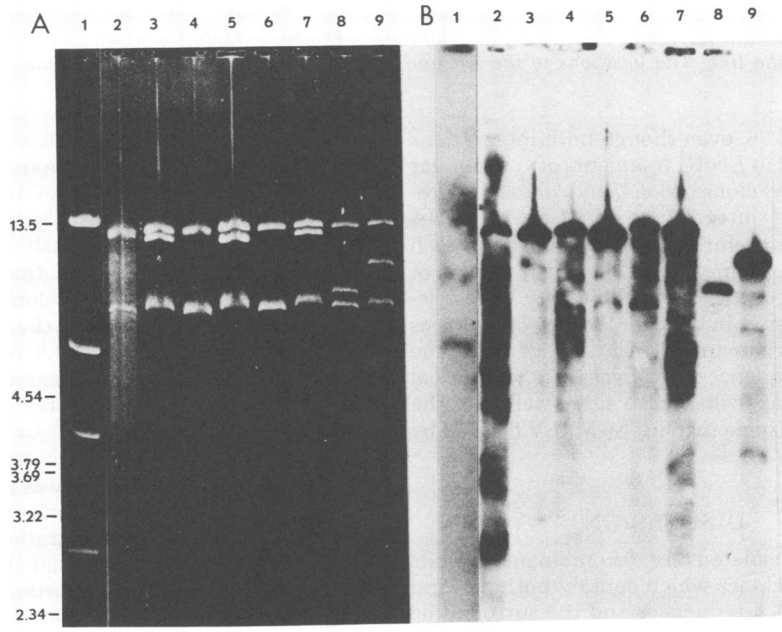

FIG. 5. *Size of cloned inserts. DNA from several recombinant DNA clones with strong sequence homology to M-MuLV was cut with EcoRI and electrophoresed through a 0.6% agarose gel. The mobilities of marker λ EcoRI DNA fragments co-electrophoresed in the same gel are indicated, along with their sizes (Md). (A) Ethidium bromide staining of the gel; (B) hybridization with M-MuLV cDNA to a blot transfer of the same gel. (Lane 1) Mixture of λ DNA cut with HindIII, detectable by ethidium bromide staining (A), and uncut M-MuLV unintegrated linear DNA, detected by hybridization with M-MuLV cDNA (B); (lane 2) Ch4A-A9-4-48; (lane 3) Ch4A-A9-4-61; (lane 4) Ch4A-A9-4-63; (lane 5) Ch4A-A9-4-73; (lane 6) Ch4A-A9-4-75; (lane 7) Ch4A-A9-4-76; (lane 8) Ch4A-A9-4-70; (lane 9) Ch4A-A9-4-27.*

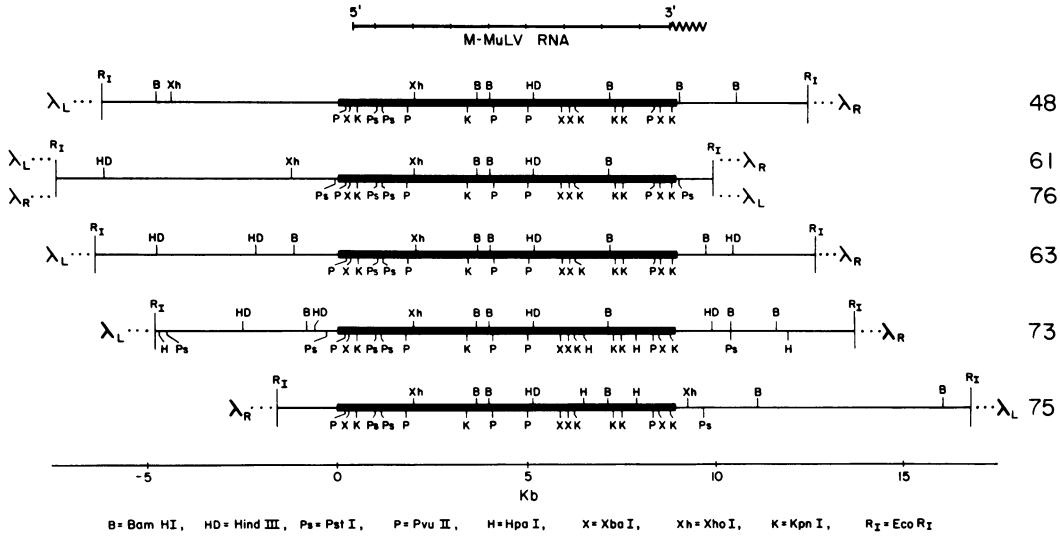

FIG. 6. *Restriction maps of M-MuLV proviral clones. Restriction maps for the six M-MuLV proviral clones are shown. For convenience of orientation, coordinates are shown with the M-MuLV proviral portions of each clone spanning from 0 to 8.9 kilobases on the map, and they are shown in the conventional orientation with respect to M-MuLV RNA. Adjacent cell sequences to the 5' side of the viral DNAs have negative map coordinates, and adjacent cell sequences to the 3' side have positive coordinates greater than 8.9 kilobases. The maps were derived by cleavage of recombinant phage DNAs with various restriction enzymes (singly or in combinations) followed by agarose gel electrophoresis. The total fragment patterns were determined by ethidium bromide staining, and those which contained viral sequences were identified by blot-transfer hybridization with M-MuLV cDNA. The abbreviations for the different enzymes are given in the figure; sites shown above the line have been mapped throughout the insert, whereas additional sites may exist for enzymes shown below the line. The locations of the left and right arms of the Charon 4A cloning vector are also indicated.*

clones 61 and 76, even though both integrations are contained in *Eco*RI fragments of very similar size. Similarly, clones 48, 63, and 75 each represent different integrations of M-MuLV DNA sequences into cellular DNA, even though each provirus is contained in an *Eco*RI fragment of 12.5 Md. In contrast to the diversity of restriction enzyme sites in the cellular DNA sequences flanking each integrated M-MuLV genome, the viral DNA sequences in each case show identical restriction maps which also agree well with the restriction enzyme map for M-MuLV derived by Gilboa et al. (13).

DISCUSSION

We have isolated six recombinant lambda phage DNA clones which contain both proviral M-MuLV DNA sequences and the surrounding cellular DNA sequences into which the viral DNA was inserted. Five different integrations of M-MuLV are represented on these clones, even though several of the *Eco*RI DNA fragments containing these integrations are of very similar size. We consider these integrations distinct because each shows a unique distribution of restriction enzyme sites in the cellular DNA sequences surrounding the proviral DNA sequences. The number of different integrations of M-MuLV present in *Eco*RI fragments of 12.0×10^6 and 12.5×10^6 daltons was initially somewhat surprising, since we had previously assumed that the new *Eco*RI fragments of this size seen in *Eco*RI digests of M-MuLV clone A9 cellular DNA were each the result of the insertion of a single M-MuLV proviral DNA sequence. The isolation of five different integrations is, however, compatible with our previous estimates that this cell line contains 10 to 12 integrated copies of M-MuLV (7). Furthermore, the fact that the same integration site was independently isolated twice among six clones suggests that most of the M-MuLV integrations in *Eco*RI fragments of this size have been cloned.

Although the cloning efforts with the M-MuLV clone A9 cell DNA successfully yielded the M-MuLV proviral clones described here, efforts to perform similar experiments with two other DNA fractions from other productively infected cells were unsuccessful. *Eco*RI fragments of 6 to 10 Md from the cell line M-MuLV clone E7 (9) which carries integrated M-MuLV DNA sequences on *Eco*RI fragments of 9.5, 8.6, 6.6, and 3.75 Md (2) were cloned, and 22,500

recombinant phage were screened. Ten phage clones were found to hybridize M-MuLV cDNA and four strongly hybridized. However, none of the clones contained a M-MuLV provirus, although some contained endogenous M-MuLV-related sequences. Similarly, *Eco*RI fragments of 6 to 10 Md from M-MuLV clone 4A cells (9) were cloned, and 2.3×10^5 recombinant phage were screened; 34 phage clones hybridized with M-MuLV cDNA, and 15 strongly hybridized. Again, none of these clones contained a genuine M-MuLV provirus. In the second experiment, the 6- to 10-Md 4A *Eco*RI fragments were digested with *Bam*HI and found to yield a 1.9-Md *Bam* fragment diagnostic of M-MuLV. In addition, this DNA gave rise to XC-positive virus upon DNA infection of NIH/3T3 cells. By these criteria, at least the M-MuLV clone 4A cell DNA fraction should have contained intact M-MuLV proviruses. Several possibilities might explain the failure to obtain M-MuLV proviral clones in two cases. (i) Not enough recombinant phage may have been screened. This may have been the case for the experiments with the M-MuLV clone E7 DNA. However, sufficient numbers of phage were screened for the M-MuLV clone 4A trials that it could be calculated that any single copy DNA sequence present should have been cloned. (ii) The particular DNA fractions chosen may have contained relatively higher concentrations of endogenous MuLV-related sequences. This was probably the case, since the 6- to 10-Md range of *Eco*RI fragments from infected or uninfected mouse cells contains many more fragments with sequence homology to M-MuLV than the 12- to 13-Md range. However, the failure to isolate a single M-MuLV proviral clone contrasts sharply with the relative ease of cloning from M-MuLV clone A9 cells. (iii) Some proviral integrations may not be clonable in bacterial systems. Evidence that certain sequences in the murine mammary tumor virus genome cannot be cloned has been obtained by several research groups (G. Hager, personal communication; H. E. Varmus, personal communication), and it is possible that some M-MuLV proviral integrations may have similar properties. In the case of M-MuLV, the putative unclonable sequences would not be located in the viral sequences themselves (since other M-MuLV proviruses were successfully cloned), but rather in particular adjacent cellular sequences. (iv) M-MuLV proviral integrations may have been cloned and subsequently lost. Instability of recombinant phage clones carrying proviral sequences has been observed for other murine retroviruses (20, 27). Retroviruses also carry sequences resembling classical procaryotic and eucaryotic transposable elements, which may result in sequence arrangement during growth (22a). Indeed, we have observed that many of the recombinant phage clones isolated (carrying both genuine M-MuLV proviruses as well as endogenous MuLV-related sequences) display instability during propagation. Growth of virus preparations is frequently accompanied by generation of deleted forms of the original phage, which can be detected by the presence of phage particles with lower buoyant density. We have found it necessary to routinely band all phage preparations in equilibrium CsCl gradients and use only the phage of greatest density to minimize these problems.

We have tested the biological activity of each M-MuLV-containing recombinant DNA clone by using the isolated phage DNA to infect NIH/3T3 cells. Each of the clones identified as containing M-MuLV based on the presence of diagnostic restriction enzyme fragments also gave rise to infectious NB tropic virus, confirming the identification of these clones. The observation that DNA from each clone was infectious differs somewhat from several other studies in which proviruses resulting from recent viral infection (as opposed to genetically transmitted endogenous proviruses) were isolated as recombinant DNA clones. Lowy et al. (20) have isolated three clones of integrated AKR viral DNA sequences from a mass culture of infected fibroblasts. One of these clones was noninfectious, whereas the other two clones were infectious, although with approximately 30-fold difference in specific infectivity. O'Rear et al. (21) have cloned integrated spleen necrosis virus DNA sequences from chronically and acutely infected chicken cells. Both infectious and noninfectious clones were obtained. Although infectious clones contained inserts of smaller size than the noninfectious integrations cloned, size did not appear to account for the difference in biological activity. Mullins et al. (J. I. Mullins, M. Nicholson, J. Casey, K. Burck and N. Davidson, personal communication) have cloned seven integrations of feline leukemia virus from an infected human cell line and obtained both infectious and noninfectious isolates. No major differences between the viral sequences of infectious and noninfectious clones could be detected. It is unclear whether the infectious nature of all of our clones is merely chance or whether M-MuLV proviruses are more often infectious than AKR, spleen necrosis virus, or feline leukemia proviruses.

The infectivity of all five of the M-MuLV proviruses cloned stands in apparent contrast to the DNase I resistance and, therefore, potential

transcriptional inactivity of many of the integrated copies of M-MuLV found in the A9 cell line. We are currently investigating the specific infectivity of individual cloned M-MuLV proviruses and the transcription activity of these individual integrations of M-MuLV in the A9 cell line.

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