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Multiple Integration Sites for Moloney Murine Leukemia Virus in Productively Infected Mouse Fibroblasts

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The integration sites for viral DNA in cells infected with Moloney murine leukemia virus (M-MuLV) were studied by restriction endonuclease cleavage of cellular DNA followed by electrophoresis in agarose gels, blot transfer to nitrocellulose, and detection of M-MuLV-related sequences by hybridization with high-specific-activity ^{32}P-labeled M-MuLV complementary DNA. When *Eco*RI was used to cleave cellular DNA, numerous DNA fragments with sequence homology to M-MuLV were detected in uninfected mouse cell DNA. These endogenous sequences are mouse specific since they are not detectable in rat cell DNA, and are related to the 38S genomic RNA of M-MuLV. Infected cells contain additional M-MuLV-specific DNA fragments which are not detected in uninfected cells. Different patterns of M-MuLV-specific DNA fragments were detected in each cloned infected line examined. These data suggest the existence of multiple sites for integration of M-MuLV DNA in infected mouse fibroblasts. Cleavage of infected cell DNA with *Bam*HI, which cleaves M-MuLV viral DNA at least twice, released the internal *Bam*HI B fragment from each infected line, confirming the presence of integrated M-MuLV DNA sequences in each infected cell line which retain some features of the sequence organization of unintegrated M-MuLV DNA.

Moloney murine leukemia virus (M-MuLV), as well as other murine and avian retroviruses, establishes a permanent association with its infected cell host in which viral DNA is stably integrated into the host chromosomal DNA. Little, however, is known about the nature or possible specificity of this integration event. Khoury and Hanafusa (17) have suggested a limited number of sites for the integration of the viral DNA of avian oncornaviruses (RAV-2) into infected chicken cell DNA. This suggestion was based on the permanent acquisition of a limited number of stably integrated proviral DNA copies despite widely differing multiplicities of infection and the transient presence of large numbers of unintegrated viral DNA molecules shortly after infection. We (7) have observed a similar acquisition of 0.5 to 4 copies of M-MuLV-specific viral DNA per diploid cell equivalent after infection of mouse fibroblasts with M-MuLV. Studies of MuLV-induced leukemogenesis in both AKR (3) and BALB/Mo (14) mice have revealed an increase in the number of integrated copies of AKR or M-MuLV viral DNA which occurs only in tumor tissue. This amplification appears to reach a finite limit at eight copies per diploid cell DNA equivalent for AKR, and four copies per diploid cell equivalent for M-MuLV.

In contrast to this indirect evidence suggesting a limited number of integration sites for retrovirus DNA in host cell DNA, direct examination of the configuration of integrated viral DNA sequences by restriction endonuclease cleavage has revealed a variety of integrated viral DNA structures, suggesting multiple sites for integration. In the case of reticuloendotheliosis virus DNA integration into chicken DNA studied by Temin and his colleagues, *Eco*RI fragments containing both viral DNA sequences, recognized by nucleic acid hybridization, and infectious DNA fragments, recognized by transfection studies, have been studied. In acutely infected cells, many different-sized *Eco*RI fragments carry viral DNA sequences and infectious viral DNA. In chronically infected chicken cells, viral DNA sequences are still detected in many sizes of *Eco*RI DNA fragments, but infectious DNA is limited to a single class of DNA fragments (16). Such results suggest that the biological function assayed by transfection studies requires some sequence arrangement not found in all classes of DNA fragments carrying viral DNA.

In this study, we report our observation of multiple integration sites for M-MuLV in infected mouse fibroblasts, based on restriction endonuclease cleavage of infected cell DNA.

MATERIALS AND METHODS

Cells. All cells were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum (Irvine Scientific). The derivation of M-MuLV clone 1 (8), other M-MuLV-infected clones (7), and G clones carrying an endogenous M-MuLV as well as the uninfected related clones (2) were previously described.

Isolation of high-molecular-weight nuclear DNA. Cells were removed from the culture dishes by treatment with EDTA and collected by centrifugation at 1,250 × *g* in a refrigerated centrifuge. Cells were suspended in Tris-buffered saline, pelleted as before, and suspended in lysing buffer (0.15 M NaCl, 0.01 M Tris [pH 7.4], 0.01 M $MgCl_2$). Nonidet P-40 (Shell) was added to a final concentration of 0.5%, the cell suspension was vigouously blended in a Vortex mixer for 30 s, and nuclei were pelleted by centrifugation for 3 min at 1,250 × *g* at 4°C. The supernatant cytoplasmic lysate was removed, and the nuclear pellet was suspended in 20 volumes of saline EDTA (0.75 M NaCl–0.25 M EDTA) by gentle pipetting. Sodium dodecyl sulfate (SDS) was added to a final concentration of 1%, and pronase (CalBiochem, B grade, predigested for 1 h at 37°C) was added to a final concentration of 100 to 200 μg/ml. The viscous nuclear lysate was incubated for 2 to 4 h at 37°C, extracted twice with an equal volume of water-saturated phenol (redistilled, pH adjusted to 7.6 to 8.0) and twice with an equal volume of chloroform containing 1% isoamyl alcohol. The aqueous layer was transferred to a clean beaker, two volumes of cold 95% ethanol was added, and the DNA was wound out. DNA was dissolved in 0.01 M Tris–0.001 M EDTA (pH 7.4)–pancreatic RNase added to a final concentration of 25 μg/ml, and the DNA was incubated for 30 min at 37°C. The solution was adjusted to 1% SDS, 0.15 M NaCl, and 50 μg of predigested pronase per ml, and incubated for an additional 30 min. The DNA was phenol extracted and chloroform extracted as before and dialyzed into 0.01 M Tris (pH 7.4).

Isolation of unintegrated proviral DNA. Subconfluent or confluent plates of 3T6 cells were treated for 1 h at 37°C with 20 μg of polybrene (Aldrich Chemical Co., Inc.) per ml in Tris-buffered saline. The polybrene was removed, and the cell monolayer was infected with M-MuLV clone 1 (8). For these infections, the source of the M-MuLV was membrane-filtered (Millipore Corp.) culture medium freshly collected from exponentially growing tissue culture dishes or roller bottles of M-MuLV clone 1 cells (typically 1 × 10^6 to 3 × 10^6 XC PFU/ml). After 20 h of incubation of culture at 37°C, the infected cells were removed from the plates by trypsinization at 4°C, washed once in cold Tris-buffered saline, and divided into nuclear and cytoplasmic fractions as described for DNA isolation. The cytoplasmic fraction was phenol and chloroform extracted as previously described (1), as were the low-molecular-weight nucleic acids from the supernatant of a Hirt extract of the nuclear fraction (12). The nucleic acids of both fractions were ethanol precipitated and redissolved in a small volume of 0.01 M Tris (pH 7.4)–0.001 M EDTA.

Synthesis of high-specific-activity M-MuLV cDNA. Complementary DNA (cDNA) was synthesized in an endogenous reaction with added calf thymus oligodeoxynucleotide primers as previously described (9), using purified M-MuLV clone 1. The virus concentration was 2.5 mg/ml, Nonidet P-40 was 0.015%, cold nucleotides were present at 1.5 mM, and α-[^{32}P]triphosphate (100 to 200 Ci/ml, ICN) was present at 5 μM. The specific activity of the synthesized cDNA was 2 × 10^8 to 4 × 10^8 cpm/μg.

Restriction enzyme digestions. Restriction enzymes were purchased from Bethesda Research Laboratories, and digestion was carried out in the recommended buffers with the addition of 0.01% gelatin (Sigma, autoclaved) to all digestion mixtures. Lambda DNA, used as a molecular weight marker, was purchased from Miles Laboratories. Typically, 10 μg of cell DNA was digested in a volume of 75 to 90 μl with 20 U of enzyme for 4 to 16 h at 37°C.

Gel electrophoresis and blot transfer. Restriction enzyme-digested DNA samples were adjusted to 0.5% SDS, 0.005 M EDTA, 10% glycerol, and 0.01% bromophenol blue by the addition of a 10×-concentrated buffer, heated to 65°C for at least 10 min, brought to 0.2% agarose, and cast in the sample wells of a horizontal 0.6% agarose gel (40 cm by 20 cm, 4 to 6 mm thick). The electrophoresis buffer was 0.04 M Tris acetate–0.005 M sodium–0.001 M EDTA (pH 7.9). Electrophoresis was carried out at 50 V for 40 to 48 h. After electrophoresis, gels were stained in 0.5-μg/ml ethidium bromide in electrophoresis buffer, and photographed under UV light (Mineralight model S-68), using an MP3 Polaroid camera with a no. 25 red 1 filter and Polaroid high-speed type 57 film. λ DNA and *Eco*RI, *Hin*dIII, and/or *Bam*HI restriction enzyme fragments of λ were run in each gel as molecular weight markers, and their positions were detected by staining with ethidium bromide prior to blot transfer. The molecular weights were assigned by the method of Cory and Adams (5). Gels were soaked for at least 45 min in 0.2 M NaOH–0.6 M NaCl, rinsed in distilled water, and soaked at least 45 min in 0.5 M Tris (pH 7.4)–0.6 M NaCl, and the DNA blot was transferred to Schleicher and Schuell BA85 nitrocellulose filter paper as described by Southern (18), using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Nitrocellulose filters were rinsed in 2× SSC, the excess solution was removed by blotting with paper towels, and the filters were dried under a heat lamp. Dried filters were baked for 4 h at 80°C in a vacuum oven.

Hybridization. Hybridizations were carried out in a buffer containing 6× SSC, 1× Denhardt's solution (5), 0.01 M *N*-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid buffer (pH 7.5), 0.1% SDS, 0.005 M EDTA, and 50 μg of yeast soluble RNA per ml at 68°C for 16 to 24 h. Filters were preincubated for 2 h at 68°C in this buffer without yeast RNA, then sealed in individual "Dazey" boilable cooking pouches, with 10 ml of hybridization buffer containing 1 × 10^6 to 2 × 10^6 cpm of ^{32} P-labeled cDNA per ml. After hybridization, filters (typically three to five filters handled at once) were rinsed in a large volume of 1× SSC at room temperature and then with 500 to 1,000 ml of 1× SSC at 68°C for 2 h, three times. Filters were then dried under a heat lamp, and autoradiography was performed using preflashed Kodak XR5 film with a tunstenate-intensifying screen (Kodak-Regular and Dupont-Lightning Plus) at −70°C for 4 to 15 days.

RESULTS

Restriction enzyme cleavage of unintegrated M-MuLV DNA molecules. To facilitate the interpretation of experiments which determined the pattern of cellular DNA fragments with sequence homology to M-MuLV, a population of unintegrated M-MuLV viral DNA molecules was first analyzed by restriction endonuclease cleavage. Mouse 3T6 cells were infected for 20 h with clone 1 M-MuLV, and total nucleic acids were purified from the cytoplasm and from the low-molecular-weight supernatant of a Hirt extract of the nuclear fraction. Aliquots of each fraction were digested with restriction endonuclease and separated by electrophoresis in 0.6% agarose gels. After electrophoresis, the DNA fragments were blot transferred to nitrocellulose filters, and those DNA molecules with sequence homology to M-MuLV were detected by hybridization with ^{32}P-labeled M-MuLV cDNA. One major virus-specific DNA species, with an apparent molecular weight of 5.8×10^6, was detected in the cytoplasm of recently infected cells (Fig. 1, track A), and presumably represents the double-stranded linear viral DNA described by Gianni et al. for M-MuLV-infected cells (11). At least five species of M-MuLV DNA molecules were detected in the nuclear fraction (Fig. 1, track A). One species comigrated with the cytoplasmic viral DNA molecule. Two rapidly migrating nuclear molecules (which can be purified by centrifugation through CsCl-ethidium bromide gradients, data not shown) presumably represent the two superhelical closed circular M-MuLV-specific DNA molecules identified in infected cells by Yoshimura and Weinberg (22), whereas the two slowly migrating molecules correspond to the relaxed circular forms. Treatment of these viral DNA preparations with *Eco*RI endonuclease failed to alter the migration of any of the M-MuLV DNA molecules, even the circular forms found in the nuclei. Thus, *Eco*RI endonuclease does not cleave M-MuLV viral DNA, as reported previously by Gianni et al. (10). Cleavage of the cytoplasmic linear DNA molecules with *Hin*dIII-D results in two smaller molecules with apparent molecular weights of 3.5×10^6 and 2.25×10^6. The nuclear DNA preparations yielded the same two fragments and in addition two larger fragments, presumably resulting from cleavage of the circular viral molecule. The larger of these two *Hin*dIII-D-cleaved molecules comigrated with the uncleaved linear cytoplasm molecule (5.8×10^6 molecular weight), whereas the smaller molecule migrated with an apparent molecular weight of 5.5×10^6. We interpret the nuclear pattern as resulting from *Hin*dIII cleavage at a single site within M-MuLV viral DNA. Circular forms, both supercoiled and relaxed, are converted to linear molecules of unit length (5.8×10^6 molecular weight) or shorter lengths, whereas linear DNA molecules are cleaved to the same subgenomic fragments generated from cytoplasmic linear viral DNA molecules. *Bam*HI cleavage generated three fragments from the cytoplasmic DNA molecule (molecular weights of 2.5×10^6, 1.95×10^6, and 1.18×10^6), whereas the nuclear

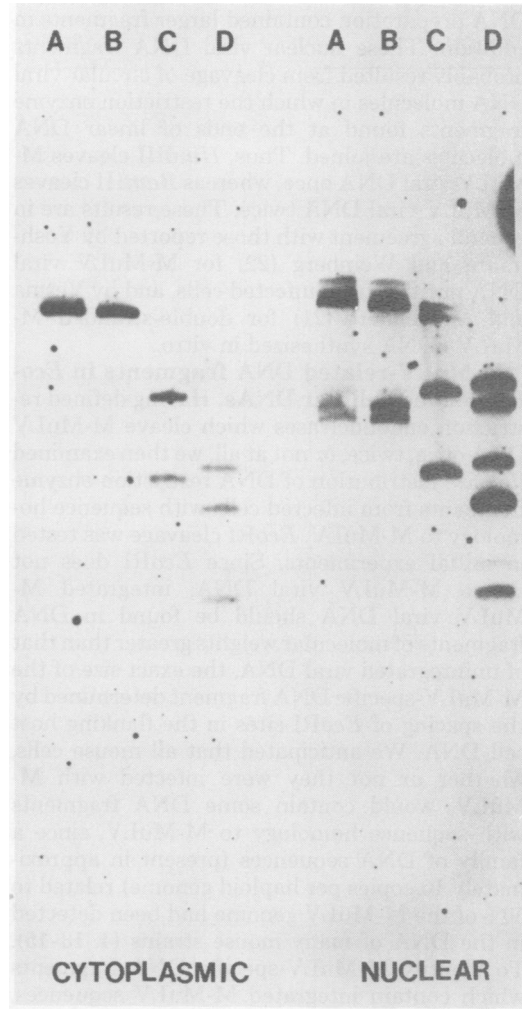

FIG. 1. *Restriction enzyme cleavage pattern of unintegrated viral DNA. Unintegrated viral DNA molecules were isolated from the nucleus and cytoplasm of 3T6 cells 20 h after infection with clone 1 M-MuLV at an approximate multiplicity of infection of 1. The nucleic acids in each fraction were digested with various restriction enzymes, fractionated by electrophoresis in 0.6% agarose gels, and transferred to nitrocellulose filters. M-MuLV-related sequences were detected by annealing with ^{32}P-labeled M-MuLV cDNA. (A) Untreated viral DNA, (B) EcoRI-treated DNA, (C) HindIII-treated DNA, (D) BamHI-treated DNA.*

DNA preparation contained larger fragments in addition. These nuclear viral DNA fragments probably resulted from cleavage of circular viral DNA molecules in which the restriction enzyme fragments found at the ends of linear DNA molecules are joined. Thus, *Hin*dIII cleaves M-MuLV viral DNA once, whereas *Bam*HI cleaves M-MuLV viral DNA twice. These results are in general agreement with those reported by Yoshimura and Weinberg (22) for M-MuLV viral DNA purified from infected cells, and by Verma and McKennett (21) for double-stranded M-MuLV cDNA synthesized in vitro.

M-MuLV-related DNA fragments in *Eco*RI-cleaved cellular DNAs. Having defined restriction endonucleases which cleave M-MuLV DNA once, twice, or not at all, we then examined the size distribution of DNA restriction enzyme fragments from infected cells with sequence homology to M-MuLV. *Eco*RI cleavage was tested in initial experiments. Since *Eco*RI does not cleave M-MuLV viral DNA, integrated M-MuLV viral DNA should be found in DNA fragments of molecular weights greater than that of unintegrated viral DNA, the exact size of the M-MuLV-specific DNA fragment determined by the spacing of *Eco*RI sites in the flanking host cell DNA. We anticipated that all mouse cells, whether or not they were infected with M-MuLV, would contain some DNA fragments with sequence homology to M-MuLV, since a family of DNA sequences (present in approximately 10 copies per haploid genome) related to 50% of the M-MuLV genome had been detected in the DNA of many mouse strains (4, 13–15). To identify M-MuLV-specific DNA fragments which contain integrated M-MuLV sequences, as a result of infection, the criterion was applied that the M-MuLV-specific DNA fragment should be present in DNA from infected cells and absent from the uninfected parent cell line. Figure 2 shows the pattern of *Eco*RI DNA fragments detected in uninfected NIH 3T3 cell DNA (track a) and M-MuLV-infected NIH 3T3 cell DNA (M-MuLV, clone A9, track b). It is apparent that a very large number of *Eco*RI DNA fragments from uninfected cell DNA have sequence homology to M-MuLV. These fragments, presumably containing the DNA sequences which hybridize to 50% of M-MuLV cDNA, include fragments both larger and smaller than unintegrated M-MuLV viral DNA. Close examination reveals several new DNA fragments detected by M-MuLV cDNA in the M-MuLV clone A9 cells. These new fragments, found only in the infected cell DNA, are tentatively identified as containing integrated M-MuLV-specific DNA sequences. The unexpectedly large number of DNA fragments with sequence homology to M-MuLV cDNA detected in uninfected cells prompted several experiments to test the specificity of our hybridization reaction. Figure 2 shows that two duplicate blots of *Eco*RI cleaved uninfected NIH 3T3 cell DNA and M-MuLV-infected clone A9 cell DNA, one hybridized under standard conditions (6× SSC, 68°C, see Materials and Methods) and the second under considerably more stringent conditions (1× SSC, 68°C) before both were subjected to the standard washing protocol. The same pattern of bands was detected in both hybridizations, indicating that the standard protocol of hybridizing under relaxed hybridization criteria (6× SSC, 68°C) did not lead to the detection of additional DNA fragments not recognized under

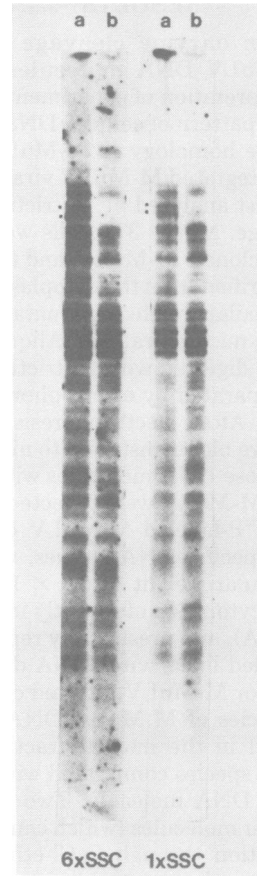

FIG. 2. *M-MuLV-related DNA fragments in EcoRI-cleaved DNA from infected and uninfected cells. (a) Clone A9 M-MuLV-infected NIH 3T3 cell DNA, (b) uninfected NIH 3T3 cell DNA. Filters were hybridized to ^{32}P-labeled cDNA in 6× SSC at 68°C or in 1× SSC at 68°C before washing (see text). Dots to the left of each track indicate additional DNA fragments found only in M-MuLV-infected cells. These fragments have apparent molecular weights of 11×10^6 and 12×10^6 to 13×10^6.*

more stringent hybridization conditions.

The M-MuLV cDNA probe used in these hybridizations was generated in an endogenous reverse transcriptase reaction, utilizing detergent-disrupted virus and exogenously added oligodeoxynucleotide primers. Thus, it was possible that an RNA packaged into virus particles (for example, cellular mRNA's) might have been transcribed into cDNA and hybridized to DNA fragments not related to M-MuLV. Figure 3 shows M-MuLV cDNA hybridization to *Eco*RI digests of infected (track d) and uninfected mouse cell DNA (tracks a and b), as well as *Eco*RI-digested rat NRK cell DNA (track c). It is apparent that the M-MuLV cDNA does not anneal significantly to rat cell DNA. Thus, all of the M-MuLV-related fragments detected in uninfected mouse cell DNA are specific to mouse cells and not found in rat cells. Rosbash et al. demonstrated that the vast majority of polyadenylic acid-positive cytoplasmic RNA sequences are conserved between mouse and rat (18). Since our M-MuLV cDNA probe failed to detect related sequences in rat DNA, this argues against the presence of cellular RNA transcripts in the M-MuLV cDNA being responsible for the multiplicity of M-MuLV-related DNA fragments detected in uninfected mouse cell DNA.

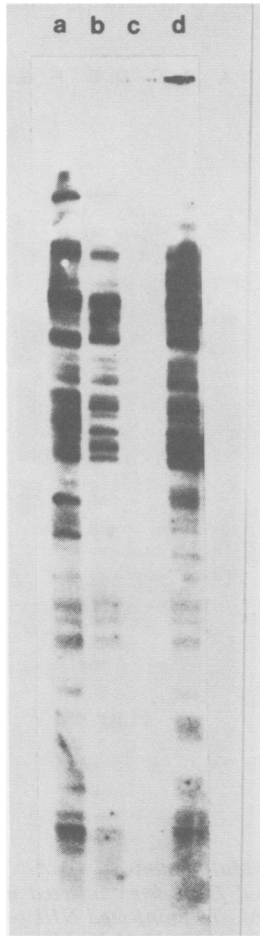

FIG. 3. *M-MuLV-related DNA fragments in EcoRI-cleaved DNA from rat and mouse cells. (A) 3T6 uninfected NIH mouse cell DNA, (B) 3T3 uninfected NIH mouse cell DNA, (C) NRK uninfected rat cell DNA, (D) clone A9 M-MuLV-infected NIH mouse cell DNA.*

A third test for specificity demonstrated that the vast majority of M-MuLV cDNA hybridization to infected and uninfected cell DNA can be prevented by the addition of purified M-MuLV RNA (Fig. 4). Figure 4, panel A, shows hybridization under normal conditions, whereas Fig. 4, panel B, is a duplicate blot hybridized to an equivalent amount of M-MuLV cDNA in the presence of purified 38S RNA from purified M-MuLV virions. The M-MuLV 38S RNA was purified by resedimentation of denatured virion 70S RNA, and only full-length RNA was used. The ability of purified viral genome RNA to compete for all of the M-MuLV cDNA hybridization argues strongly that all of the DNA fragments detected in an *Eco*RI digest of uninfected cell DNA are in fact related by DNA sequence to M-MuLV. Finally, we have used a M-MuLV cDNA probe generated using virus grown in rat cells. The rat cell line was infected at endpoint dilution with M-MuLV. It is highly unlikely that this new cell line carries mouse-specific sequences, including the endogenous virus-like 30S RNA which is only poorly infectious. Such a cDNA probe detects the same pattern of *Eco*RI DNA fragments in mouse cell DNA as does M-MuLV clone 1 cDNA probe (data not shown).

Multiple *Eco*RI fragments containing integrated M-MuLV DNA sequences. A number of independent M-MuLV-infected and cloned mouse fibroblast lines were examined and compared to their uninfected parent cell lines. Figure 5 shows the M-MuLV-related fragments detected in *Eco*RI digests of a number of NIH Swiss 3T3 cell lines infected with M-MuLV. In each infected line at least one new M-MuLV-specific DNA fragment not found in uninfected cells was detected. For example, M-MuLV clone 4A has a new fragment of molecular weight 16.5×10^6. Clone A9 contained new DNA fragments at molecular weights of 28×10^6 to 32×10^6, 21×10^6, 12×10^6, and 8.7×10^6. Similarly, M-MuLV clone E7 carries a new *Eco*RI DNA fragment of molecular weight 18.5×10^6 which is detected by M-MuLV cDNA, and M-MuLV clone 1 contains M-MuLV-specific *Eco*RI fragments of 30×10^6, 21×10^6, and 17.5×10^6 to 18×10^6. Figure 6 shows a number of cloned lines of M-MuLV-infected BALB SVT2 cells and

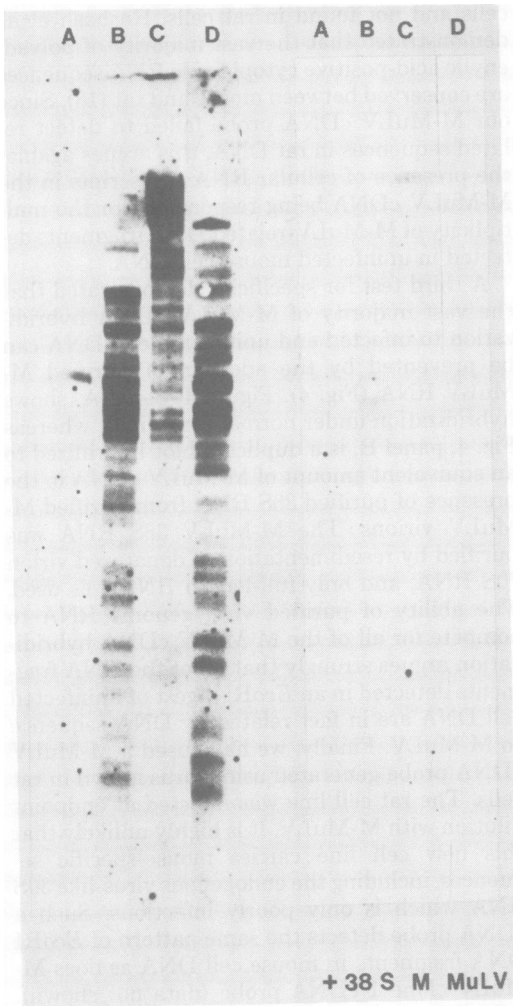

FIG. 4. *Competition for hybridization of ^{32}P-labeled cDNA to M-MuLV-related DNA fragments in EcoRI-cleaved mouse cell DNA by cold M-MuLV viral RNA. ^{32}P-labeled M-MuLV cDNA was hybridized to duplicate nitrocellulose filters in the presence or absence of a 20-fold weight excess of purified, full-length viral 38S RNA. (A) Unintegrated viral DNA, (B) G clone 1 cell DNA, (C) clone A9 M-MuLV-infected NIH cell DNA, (D) 3T3 uninfected NIH cell DNA.*

compares the pattern of M-MuLV-related fragments in each infected line to that of the uninfected SVT2 parent line. As before, at least one new M-MuLV-related DNA fragment was identified in each infected line. Finally, Fig. 7 shows the M-MuLV-related *Eco*RI DNA fragments detected in two clones of G cells, a fibroblast cell line derived from the BALB/Mo strain of mice developed by Jaenisch (13) which carry a single, genetically transmitted M-MuLV genome as an endogenous virus (2). A single new M-MuLV *Eco*RI fragment of approximately 16.5 × 10^6 molecular weight is seen in these cells that is not detected in the DNA of H cells, H cells being derived from a sibling embryo to the embryo giving rise to the G cell line which did not inherit the genetically transmitted M-MuLV genome. Table 1 summarizes the estimated molecular weight of M-MuLV-specific fragments found in the different infected lines studied. At least nine different M-MuLV-specific *Eco*RI fragments have been identified in infected NIH cell lines, whereas a total of six have been identified in BALB/c cells, either infected with or genetically transmitting M-MuLV. The molecular weight of each M-MuLV-specific DNA fragment is unique. None of the M-MuLV-specific fragments in any of the cell lines examined had the same molecular weight.

A second line of evidence confirming the pres-

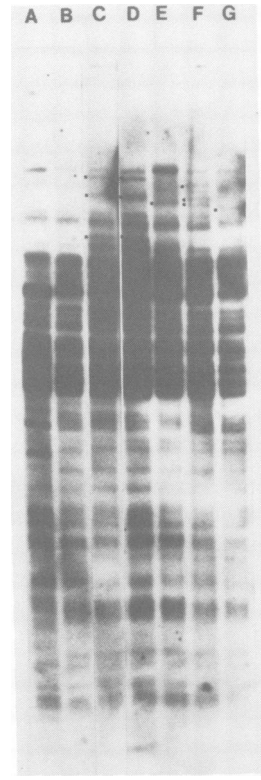

FIG. 5. *M-MuLV-related DNA fragments in EcoRI-cleaved DNA from infected and uninfected NIH cells. (A) 3T6 uninfected NIH cell, (B) 3T3 uninfected NIH cell, (C) A9 M-MuLV-infected NIH cell, (D) A9 M-MuLV-infected NIH cell, (E) E7 M-MuLV-infected NIH cell, (F) clone 1 M-MuLV-infected NIH cell, (G) clone 4A M-MuLV-infected NIH cell. Dots to the left of each track indicate additional DNA fragments found only in M-MuLV-infected cells.*

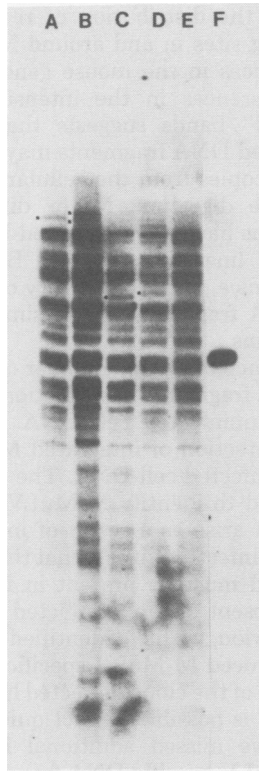

FIG. 6. *M-MuLV-related DNA fragments in EcoRI-cleaved DNA from infected and uninfected BALB/c SVT2 cells. (A) D2 M-MuLV-infected BALB/c SVT2 cells, (B) D2-B M-MuLV-infected BALB/c SVT2 cells, (C) D2-C M-MuLV-infected BALB/c SVT2 cells, (D) D2-D M-MuLV-infected BALB/c SVT2 cells, (E) SVT2 uninfected BALB/c SVT2, (F) unintegrated viral DNA. Dots to the left of each track indicate additional DNA fragments found only in M-MuLV-infected cells.*

ence of integrated M-MuLV proviral DNA in each infected cell line comes from an examination of the pattern of M-MuLV-related fragments generated by *Bam*HI cleavage of infected cell DNA. *Bam*HI cleaves unintegrated M-MuLV DNA at least twice, generating three DNA fragments of approximate molecular weights as follows: A = 2.5×10^6, B = 1.9×10^6, and C = 1.25×10^6, as shown above. Mapping studies by Verma and McKennett (21) and Yoshimura and Weinberg (22) have identified the order of these fragments as 5'—A, B, C—3'. Thus, the 1.9×10^6-molecular weight B fragment is an internal fragment, flanked on both sides by viral DNA sequences. Figure 8 demonstrates that cleavage of each of the M-MuLV-infected cell DNAs with *Bam*HI released a 1.9×10^6-molecular weight M-MuLV-specific fragment which comigrated with the M-MuLV *Bam*HI B fragment. No M-MuLV-related fragment of similar molecular weight was released from uninfected NIH 3T3 cell DNA, BALB/c 3T3 cell DNA, or H cell DNA (BALB/c derived, Mov-1 locus negative). Thus, each infected cell line contains an integrated M-MuLV DNA arrangement similar enough to that found in unintegrated M-MuLV viral DNA to allow the production of a *Bam*HI B fragment. Fragments from infected cells comigrating with the *Bam*HI A and C fragments are not found, although this observation is tentative, due to the presence of M-MuLV DNA fragments from uninfected cells

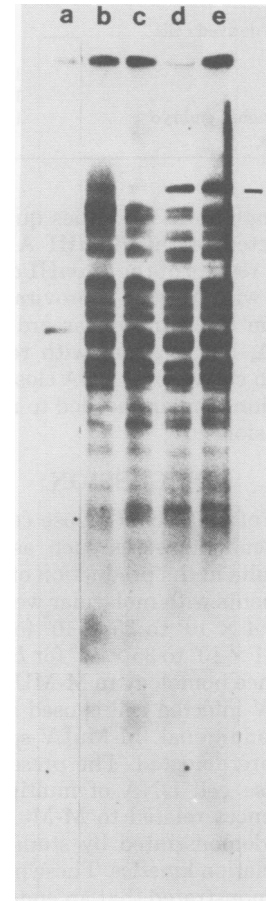

FIG. 7. *M-MuLV-related DNA fragments in EcoRI-cleaved DNA from uninfected BALB/c cells and cells from mice genetically transmitting M-MuLV. (a) Unintegrated cytoplasmic M-MuLV viral DNA (5.8×10^6); (b) A 31-uninfected BALB/c 3T3 cell; (c) H, uninfected BALB/c cell; (d) G clone 1, cloned cell line from mice genetically transmitting M-MuLV (reference 2); (e) G clone 3, second cloned cell line from mice genetically transmitting M-MuLV. The bar indicates the new 16.5×10^6-molecular weight M-MuLV-specific DNA fragment found only in cell lines from a strain of mice genetically transmitting M-MuLV.*

TABLE 1. *Molecular weight of M-MuLV-specific DNA fragments in EcoRI-cleaved cellular DNA*

Source of DNA	Apparent mol wt ($\times 10^6$)
Unintegrated cytoplasmic viral DNA	5.75
M-MuLV-infected NIH cells	
Clone 1	30, 20-21, 17.5-18
Clone 4A	16.5
Clone A9	28-32, 21-22, 12-13, 8.7-9
Clone E7	18.5
M-MuLV-infected BALB SVT2 cells	
Clone D2	17.6
Clone D2-B	18.0, 16.7
Clone D2-C	8.0
Clone D2-D	8.3
BALB/c (Mo) derived cell lines	
G clone 1	16.5-17
G clone 3	16.5-17
Secondary mouse embryo fibroblasts	16.5-17

with electrophoretic mobilities quite similar to those expected of the *Bam*HI A and C fragments. The results of the *Bam*HI digestions are compatible with M-MuLV proviral DNA being integrated in the same linear order as the genomic RNA, but analyses with restriction enzymes which cleave viral DNA closer to the cell-viral DNA junction are needed to make a definitive conclusion.

DISCUSSION

Cleavage of uninfected mouse cell DNA with restriction endonucleases such as *Eco*RI and *Bam*HI results in the production of a number of DNA fragments with molecular weights ranging from about 4×10^6 to 35×10^6 for *Eco*RI and from about 1×10^6 to 35×10^6 for *Bam*HI which have sequence homology to M-MULV cDNA. If an M-MuLV-infected cell is used as the source of DNA, additional M-MuLV-specific DNA fragments are detected. The presence in uninfected mouse cell DNA of multiple copies of DNA sequences related to M-MuLV has been previously demonstrated by studies of nucleic acid reassociation kinetics. These previous studies have demonstrated that an endogenous family of DNA sequences (composed of about 10 members per haploid genome) is present in many mouse strains and has sequence homology to approximately 50% of the M-MuLV viral genome. Our M-MuLV cDNA probe reacts with these endogenous "related" sequences as well as with M-MuLV-specific sequences, and we have demonstrated that a large number of M-MuLV-related DNA restriction enzyme fragments are detected, even in cells not infected with M-MuLV. The many M-MuLV-related DNA fragments seen in uninfected mouse cells presumably reflect the distribution of restriction enzyme cutting sites in and around M-MuLV-related sequences in the mouse genome. Reproducible differences in the intensity of these "background" bands suggests that some M-MuLV-related DNA fragments may be released in multiple copies from the cellular DNA upon endonuclease digestion. Minor differences in band patterns have been observed between uninfected cell lines derived from BALB/c and NIH Swiss mice, but the majority of M-MuLV-related DNA fragments are of similar sizes in the two strains.

The presence of a large number of M-MuLV-related DNA fragments in restriction endonuclease-cleaved uninfected cell DNA has complicated the detection of integrated M-MuLV sequences in infected cell DNA. The criterion we have adopted to identify M-MuLV DNA fragments which arise as a result of integration of exogenously infecting virus is that the M-MuLV-specific band must be present in infected cell DNA and absent in the uninfected parent line. By this criterion, we have identified at least one infection-induced M-MuLV-specific DNA fragment in each of the cloned infected lines we have examined. It is possible, in fact quite probable, that we have missed additional infection-induced M-MuLV-specific DNA fragments whose molecular weights are similar to those of the endogenous M-MuLV-related sequences. For example, most of the M-MuLV-specific DNA fragments we have detected range in molecular weight from 11×10^6 to 30×10^6, a range of DNA fragment size which is relatively deficient in uninfected cell M-MuLV-related DNA fragments. Relatively few infection-specific M-MuLV DNA fragments have been detected in the molecular weight range of 6×10^6 to 11×10^6, which is nearly saturated with uninfected cell M-MuLV-related bands.

Despite the problems presented by a complex pattern of M-MuLV-related DNA fragments generated from uninfected cell DNA, a number of different-sized M-MuLV-specific DNA fragments unique to infected cells have been identified. Nine different M-MuLV-specific *Eco*RI fragments have been identified in infected NIH cells, and five more, with different molecular weights, have been identified in BALB/c-derived cells. The M-MuLV-specific fragments appear to be different in each of the cloned lines we have examined. Since the spacing of *Eco*RI recognition sites around integrated M-MuLV DNA appears to be different in each cell line, we conclude that there are many possible sites for M-MuLV in infected mouse fibroblasts. The fact that two independently derived M-MuLV-specific DNA fragments with the same molecu-

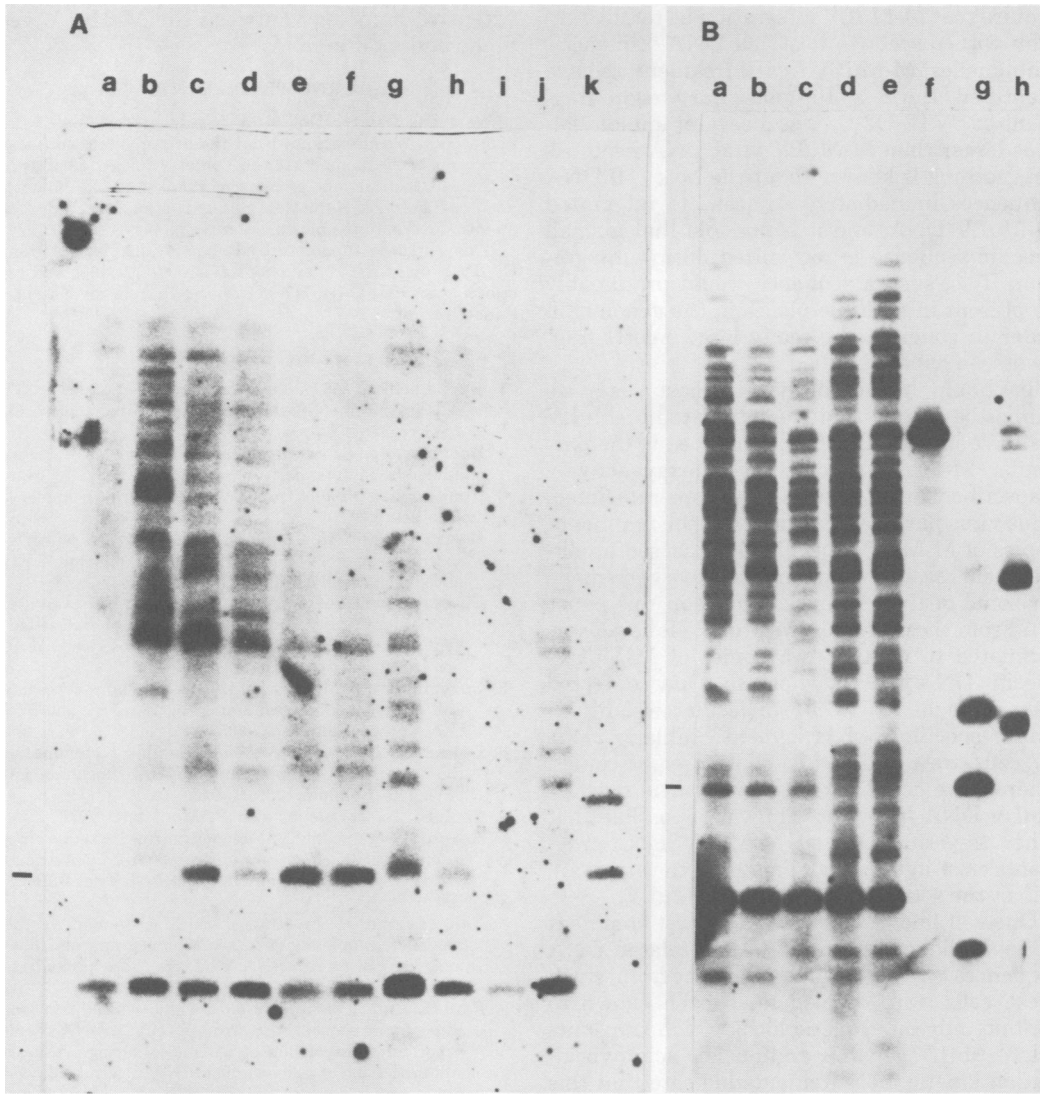

FIG. 8. *M-MuLV-related DNA fragments in BamHI-cleaved DNA from infected and uninfected cells. The position of the BamHI B fragment of unintegrated M-MuLV DNA is indicated by a bar. (A) a, NIH 3T3 uninfected NIH 3T3; b, NIH 3T6 uninfected NIH 3T3; c, clone 1 M-MuLV-infected NIH 3T3; d, clone 4A M-MuLV-infected NIH 3T3; e, clone A9 M-MuLV-infected NIH 3T3; f, clone A9 M-MuLV-infected NIH 3T3; g, G clone 1 cell line from BALB/Mo mouse carrying an endogenous M-MuLV; h, G clone 3 cell line from BALB/Mo mouse carrying an endogenous M-MuLV; i, G clone 8 cell line from BALB/Mo mouse carrying an endogenous M-MuLV (this line has lost the M-MuLV DNA sequences); j, cell line from BALB/c mice; k, unintegrated viral DNA. (B) a, Clone 4A M-MuLV-infected NIH 3T3; b, clone 3A M-MuLV-infected NIH 3T3; c, G clone 1 cell line from BALB/Mo mouse carrying an endogenous M-MuLV; d, H uninfected BALB/c cells; e, H3 1 uninfected BALB/c cells; f, unintegrated viral DNA EcoRI-treated; g, unintegrated viral DNA BamHI-treated; h, unintegrated viral DNA HindIII-treated.*

lar weight were not observed among the 15 different M-MuLV DNA integrations detected here indicates that the number of possible integration sites is quite large. One possible interpretation of our data is that integration of M-MuLV occurs at random sites in the host cell DNA, although the results of *Bam*HI digestions indicate that insertion is not random with respect to the order of viral DNA sequences. However, the demonstration of multiple integration sites, as defined by the spacing of *Eco*RI sites adjacent to integrated M-MuLV DNA, does not

require that M-MuLV integration be totally random with respect to host cell DNA sequences. The smallest M-MuLV *Eco*RI fragment we have identified, the 8×10^6-molecular weight fragment of SVT2 D2-C, is still several million daltons larger than M-MuLV viral DNA itself. As yet, nothing is known about the host cell DNA sequences immediately adjacent to integrated M-MuLV DNA, and it is possible that a small specific sequence is recognized during integration. If so, such a sequence would presumably be present in multiple places in the genome, in order to generate the many large *Eco*RI fragments we have identified.

It should be noted that in most cases we cannot be certain that the integrated M-MuLV we have identified is functional, i.e., is the particular M-MuLV-specific DNA array actively transcribed in infected cells to generate infectious virus particles. Estimates of the number of copies of M-MuLV-specific DNA, based on nucleic acid reassociation kinetics, have been made for some of the cell lines studied in this paper (7). From these studies, M-MuLV clone 4A was estimated to contain two copies of M-MuLV-specific DNA per cell, whereas we have detected only a single 16.5×10^6-molecular weight M-MuLV-specific *Eco*RI fragment. Similarly, clone E7 cells are estimated to contain eight copies, whereas we could detect only a single new M-MuLV DNA fragment. Thus, it is possible that other as-yet-undetected M-MuLV DNA fragments exist in these cell lines, and that they are in fact the source of infectious M-MuLV.

One cell line in which we are most confident of having identified all M-MuLV-related DNA sequences is the G cell line. The mice from which these cells were derived have been shown to contain only one genetic locus which transmits the M-MuLV genome, and nucleic acid hybridization kinetic measurements indicate that this locus contains only one copy of M-MuLV DNA. It is thus likely that the 16.5×10^6-molecular weight M-MuLV-specific DNA fragment detected in the G cells (Fig. 7) represents this integrated M-MuLV DNA. In agreement with this, Anton Berns (personal communication) has studied DNA from the same animals by *Eco*RI digestion, gel electrophoresis, and hybridization in solution with M-MuLV cDNA rendered free of sequences which recognize uninfected cell DNA. By this different method, a M-MuLV-specific DNA fragment of similar size was detected.

Steffen and Weinberg (20) recently described similar experiments studying the integrated M-MuLV cDNA sequences found in rat and mouse cells. They also observed multiple integration sites in both mouse and rat cells, as well as the extensive homology between the M-MuLV genome and mouse DNA.

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