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Methylation State and DNase I Sensitivity of Chromatin Containing Moloney Murine Leukemia Virus DNA in Exogenously Infected Mouse Cells

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The nature of Moloney murine leukemia virus (M-MuLV)-specific proviral DNA in exogenously infected mouse cells was studied. M-MuLV clone A9 cells, NIH-3T3 fibroblasts productively infected with M-MuLV, were used. These cells contain 10 to 15 copies of M-MuLV proviral DNA. The state of methylation of M-MuLV proviral DNA was examined by cleaving A9 cell DNA with restriction endonucleases which have the dinucleotide CpG in their cleavage sequences. Analysis with such enzymes, which recognized nine different sites in M-MuLV DNA, indicated that most if not all of the M-MuLV proviruses in A9 cells were completely unmethylated. An individual proviral integration was examined, using as probe adjacent single-copy cellular sequences. These sequences were obtained from a lambda phage recombinant clone containing an M-MuLV provirus from the A9 cells. This individual integration also showed no detectable methylation. In contrast, endogenous MuLV-related sequences present in NIH-3T3 cells before infection were largely methylated. The configuration of chromatin containing M-MuLV proviruses was also investigated by digesting A9 nuclei with DNase I, followed by restriction analysis of the remaining DNA. Endogenous MuLV-related DNA was in chromatin relatively resistant to DNase I digestion, whereas the majority of M-MuLV-specific proviruses were in domains of intermediate DNase I sensitivity. Two proviral copies hypersensitive to DNase I digestion were identified. Analogy to the DNase I sensitivity of expressed and nonexpressed globin genes suggested that the proviral copies containing DNase I-hypersensitive sites were transcribed.

Moloney murine leukemia virus (M-MuLV), like all retroviruses, replicates via a double-stranded DNA intermediate (34). The double-stranded DNA molecule is synthesized in the cytoplasm as a linear molecule which is colinear with viral RNA and contains terminal repetitions (the long terminal repeats [LTRs]; 20, 28, 33). This DNA is then transported to the nucleus of the cell, where it integrates into the chromosomal DNA to form the provirus. Viral RNA is synthesized from proviral DNA by cellular RNA polymerase II, the enzyme which synthesizes cellular heterogeneous nuclear RNA (hnRNA) and mRNA. The structure and specificity of proviral DNA integration have been studied in great detail (reviewed by H. E. Varmus and R. Swanstrom [38]). Integration of proviral DNA occurs at many sites within the host chromosomal DNA. The viral DNA is not permuted during integration, and thus it contains LTRs at either end.

Proviral DNA represents a complete transcription unit. The LTRs contain the signals required for initiation of viral RNA synthesis (15, 36), as well as for cleavage-polyadenylation (29, 36). Transcription presumably begins in the 5′ LTR and terminates in the 3′ LTR. The size of the initial transcript of M-MuLV RNA in infected cells is the same size as mature virion RNA (12). This provides a very useful system for studying the effect of adjacent cell sequences on expression of a gene ("position effect"), since integration of the proviral transcription unit occurs at multiple sites within the host chromosomal DNA.

Several lines of evidence indicate that adjacent host cell DNA sequences or chromatin configuration can influence the transcriptional activity of retrovirus proviral DNA. Infection of M-MuLV in embryonic mice has been studied by Jaenisch et al. (21). A variety of patterns of expression in different tissues was observed

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after birth, and the patterns were different for different proviral integrations. Ringold et al. (27) studied rat hepatoma cells infected in vitro with murine mammary tumor virus and found that some cells into which murine mammary tumor virus proviral DNA had integrated expressed viral RNA, whereas others did not. We studied the transcriptional activity of M-MuLV proviral DNA in exogenously infected mouse fibroblasts by the technique of DNase I digestion of chromatin (5). Nuclei from cell lines which contained multiple copies of integrated M-MuLV proviral DNA were digested with DNase I, and the relative sensitivity of M-MuLV-specific DNA was measured by solution hybridization. The results indicated that only a minority of M-MuLV-specific DNA was preferentially sensitive to DNase I digestion. Weintraub and Groudine (41) have shown that transcribed genes are preferentially sensitive to DNase I digestion, and our results suggested that the majority of integrated M-MuLV DNA copies in exogenously infected cells are not transcribed.

In the experiments reported here, a cloned line of NIH-3T3 cells productively infected with M-MuLV was studied. The state of methylation of the M-MuLV proviral DNA was investigated, since several studies have shown that transcribed genes are usually undermethylated (7, 22, 37). In addition, proviral integrations were examined for the presence of sites of DNase I hypersensitivity, which are indicative of transcribed genes (31, 44). The results showed that endogenous MuLV-related DNA was relatively methylated and was contained within chromatin resistant to DNase I. In contrast, M-MuLV proviral copies were unmethylated and were in chromatin of intermediate DNase I sensitivity. Also, two M-MuLV proviruses were found to be in chromatin which was very sensitive to DNase I and thus contained sites of DNase I hypersensitivity.

MATERIALS AND METHODS

Cells. M-MuLV clone A9 cells are a line of NIH-3T3 cells productively infected with M-MuLV and have been described previously (13). They were grown in tissue culture monolayers in Dulbecco-modified Eagle medium supplemented with 10% calf serum (Irvine Scientific). NIH-3T3 cells (35) were grown in the same medium.

Cell fractionation and DNase I digestion of nuclei. Cells were removed from tissue culture dishes by trypsinization on ice (12). For DNase I digestion experiments, cultures at one-half to three-quarters confluency were used, whereas completely confluent cultures were used for isolation of total high-molecular-weight DNA. Trypsinized cells were harvested by centrifugation (1,000 × *g* for 2 min) and washed twice in Tris-buffered saline containing calcium and magnesium. Nuclei were isolated essentially according to Weintraub and Groudine (41). A total of 2×10^8 to 3×10^8 cells were suspended in 1 ml of reticulocyte standard buffer (0.01 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], 3 mM $MgCl_2$), and Nonidet P-40 was added to 0.5%. After blending in a Vortex mixer for 30 s, the nuclei were harvested by centrifugation (1,000 × *g* for 2 min) and washed twice with reticulocyte standard buffer.

For DNase I digestion of nuclei, a modification of the conditions of Weintraub and Groudine was used. Nuclei were suspended in reticulocyte standard buffer at 25 absorbance units at 260 nm per ml and divided into several portions. DNase I (RNase-free; Worthington Diagnostics) was added to different concentrations, and the samples were incubated for 20 min at 15°C. After incubation, the reactions were terminated by adjusting the samples to 0.2 M NaCl–0.5% sodium dodecyl sulfate–10 mM EDTA–200 μg of pronase per ml (preincubated at 37°C to remove residual nucleases). The samples were then incubated overnight at room temperature or for 1 h at 37°C. High-molecular-weight DNA was then extracted.

DNA extraction. High-molecular-weight DNA was extracted by two phenol-chloroform extractions followed by two chloroform extractions, as described previously (1). The extracted DNA was precipitated by addition of 2 volumes of isopropanol, harvested by centrifugation, and suspended in 10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA.

Restriction enzyme digestion and gel electrophoresis. DNA samples were digested with various restriction enzymes (Bethesda Research Laboratories or Boehringer Mannheim Corp.) in the recommended buffers at a DNA concentration of 60 to 120 μg/ml, with the addition of gelatin to 0.01%. In experiments with methyl-sensitive enzymes, adenovirus type 2 DNA was added to the cellular DNA before digestion, and completeness of digestion was assessed by the presence of the predicted restriction fragments after gel electrophoresis, staining in ethidium bromide, and visualization with UV light.

Electrophoresis of DNA in horizontal agarose gels was performed as described previously (1). For gels which were hybridized with radioactive DNA in situ, the thickness of the gels was 3 to 4 mm. After electrophoresis, gels were stained with ethidium bromide, and distribution of DNA was determined by observation under UV light.

Preparation of labeled DNA probes. ^{32}P-labeled M-MuLV cDNA was prepared as described previously (14). Calf thymus oligodeoxynucleotide primers were added to the reaction to render the cDNA uniformly representative of the viral genome.

Nick-translation of recombinant DNA restriction fragments was performed as described by Rigby et al. (26).

Gel hybridization. For in situ hybridization to agarose gels (30), the following procedure was used. The gels were soaked for 30 min in 0.2 N NaOH–0.6 M NaCl, rinsed briefly in water, and then soaked in 0.5 M Tris-hydrochloride (pH 7.5)–0.6 M NaCl for 30 min. After rinsing the gels in water, they were dried under vacuum and gentle heat on Whatman 3MM filter paper. After drying, the gels were removed from the paper by placing them in water, and the slightly rehydrated gels were transferred to plastic food bags for hybridization.

Hybridizations were done in sealable plastic bags, as described previously (1). The gels were first prehybridized for 30 min at 68°C with prehybridization

buffer: 6× SSC = 0.9 M NaCl, 0.09 M sodium citrate; 5× Denhardt solution (9); 10 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 7.5), 500 μg of denatured salmon sperm DNA per ml, 0.1% sodium dodecyl sulfate. After prehybridization the buffer was expelled and labeled DNA probe was added in hybridization buffer (6× SSC, 1× Denhardt solution, 10 mM TES [pH 7.5], 50 μg of denatured salmon sperm DNA per ml, 0.1% sodium dodecyl sulfate), and hybridization was carried out at 68°C overnight. Concentration of the labeled cDNA was 0.5×10^6 to 1.0×10^6 cpm/ml.

After hybridization, the gels were removed from the food bags and washed briefly three times with 2× SSC + 0.1% sodium dodecyl sulfate, followed by two washes with 0.1× SSC + 0.1% sodium dodecyl sulfate for 30 min at 45 to 55°C. After washing, damp gels were sealed between two layers of cellophane wrap and exposed to X-ray film between two intensifying screens. The film was sensitized by preflashing unless otherwise noted.

RESULTS

Methylation state of M-MuLV proviral DNA. The methylation state of M-MuLV proviral DNA was investigated by using methyl-sensitive restriction endonucleases. 5-Methylcytosine is the most common methylated base found in the DNA of higher eucaryotes and is almost exclusively found in the dinucleotide CpG (18). Certain restriction endonucleases recognize and cleave a DNA sequence that contains CpG, and methylation of this dinucleotide inhibits cleavage by many (25). For these experiments, such enzymes used were *Xho*I, *Sal*I, *Cla*I, and *Sma*I since they also cut M-MuLV DNA at least once (17). The sites of cleavage for these enzymes within M-MuLV DNA are shown in Fig. 1. Two other restriction enzymes were also used, *Bam*HI and *Eco*RI. These enzymes recognize sites on the DNA that do not contain CpG and cleave methylated and unmethylated eucaryotic DNA equally well. Also, *Eco*RI does not cleave within M-MuLV DNA at all (16, 39).

***Eco*RI fragments which contain M-MuLV provirus.** In the first experiments, the methylation state of M-MuLV proviral DNA was investigated by digesting infected and uninfected cell DNA with *Eco*RI in combination with methyl-sensitive enzymes. The infected cell line M-MuLV clone A9 (13) was used for these experiments; M-MuLV clone A9 cells are NIH-3T3 fibroblasts productively infected with M-MuLV and contain 10 to 15 copies of integrated M-MuLV proviral DNA (2). Since *Eco*RI does not cleave M-MuLV DNA, digestion of infected cell DNA with *Eco*RI yields each M-MuLV provirus in a single restriction fragment, containing both M-MuLV DNA and adjacent host cell DNA sequences. In Fig. 2 (lanes 1 and 2), *Eco*RI-digested uninfected NIH-3T3 cell and A9 DNAs were separated according to size by agarose gel electrophoresis and blot-transferred to activated aminophenylthioether paper (11; B. Seed, personal communication), and restriction fragments which could hybridize radioactive M-MuLV cDNA were identified by autoradiography. The MuLV-related DNA fragments from the uninfected cells (lane 1) represented endogenous MuLVs resident in the genomes of mice, and the M-MuLV proviral copies acquired by infection were evident as additional M-MuLV-specific *Eco*RI fragments in the infected cells (1). A9 cells contain M-MuLV proviral DNA in *Eco*RI

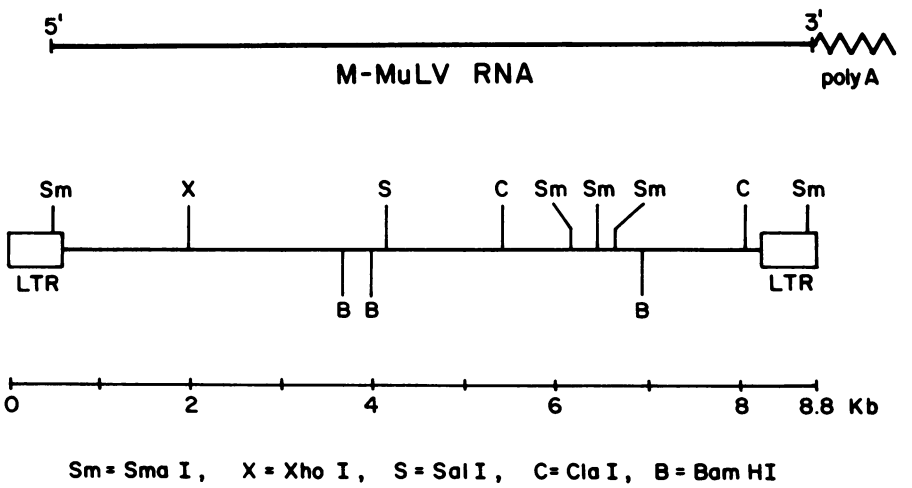


FIG. 1. Restriction map of M-MuLV DNA. A restriction map of M-MuLV DNA is shown, according to Gilboa et al. (17). The viral DNA is shown in proviral form, with two copies of the LTRs, and the linear relationship to genomic RNA is indicated. Sites for the following enzymes are indicated: B, *Bam*HI; X, *Xho*I; S, *Sal*I; C, *Cla*I; Sm, *Sma*I. The identification of the two *Cla*I sites was obtained from our own data (not shown) and confirmed in the published nucleotide sequence of M-MuLV DNA (29). Poly A, Polyadenylate.

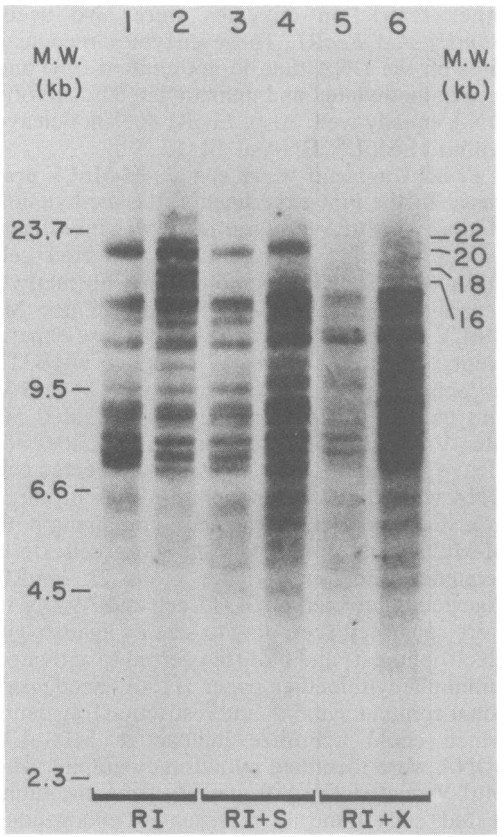


FIG. 2. Methylation of *Eco*RI fragments containing M-MuLV proviral DNA. NIH-3T3 and A9 cell DNAs were digested with *Eco*RI or *Eco*RI in combination with *Sal*I or with *Xho*I (30 μg of DNA per digest). After digestion, the DNA was resolved by electrophoresis in a 0.6% agarose gel, transferred to activated aminophenylthioester paper, and hybridized with ^{32}P-labeled M-MuLV cDNA in hybridization buffer containing 10% dextran sulfate (40). cDNA probe was at 10^6 cpm/ml. An autoradiogram (2-day exposure) of the washed blot is shown. Odd-numbered lanes are NIH-3T3 DNA, and even-numbered lanes are A9 DNA. Lanes 1 and 2, Digestion with *Eco*RI; lanes 3 and 4, digestion with *Eco*RI and *Sal*I; lanes 5 and 6, digestion with *Eco*RI and *Xho*I. M.W., Molecular weight.

fragments of 22, 20, 18, and 16 kilobases (kb; 2) (lane 2). Each of these fragment sizes consists of more than a single M-MuLV provirus. Three different 22-kb proviral organizations and two 20-kb organizations have been isolated from the A9 cells by recombinant DNA techniques (3).

In Fig. 2, lanes 3 and 4, the same DNA samples were digested with *Eco*RI in combination with *Sal*I. The *Eco*RI fragments containing integrated M-MuLV provirus were digested by the addition of *Sal*I (lane 4), but the endogenous MuLV-related fragments in the same cells were unaffected. Thus, at least one *Sal*I site was unmethylated in the *Eco*RI fragments containing each exogenously acquired M-MuLV provirus, whereas there were few unmethylated *Sal*I sites in *Eco*RI fragments carrying endogenous MuLV-related sequences. The resistance to digestion of the endogenous MuLV-containing *Eco*RI fragments was not due to a lack of *Sal*I sites, since *Sal*I sites have been identified in a number of endogenous MuLV organizations isolated by recombinant DNA techniques (H. Fan, unpublished data; R. Mural et al., personal communication).

In Fig. 2, lanes 5 and 6, uninfected and infected cell DNAs were digested with *Eco*RI in combination with *Xho*I, and the results obtained were similar to those for *Eco*RI in combination with *Sal*I, except that some of the larger endogenous *Eco*RI fragments were also digested with *Xho*I.

These results indicated that all detectable *Eco*RI fragments carrying integrated M-MuLV provirus contained unmethylated *Sal*I and *Xho*I sites. The unmethylated sites might be the *Sal*I and *Xho*I sites within the viral DNA itself, or they might be in the adjacent cell DNA on the same *Eco*RI fragment. Furthermore, methylated M-MuLV proviruses would not have been detected if they were contained in *Eco*RI fragments which comigrated with an endogenous MuLV-containing *Eco*RI fragment. For these reasons, a different approach was taken to measure methylation within the M-MuLV viral sequences themselves.

Extent of methylation within proviral DNA. The extent of methylation in M-MuLV proviral DNA copies in the A9 cells was assessed by comparing the efficiency with which methyl-sensitive and methyl-insensitive enzymes could cleave this DNA. In Fig. 3A, such an experiment was performed by cleaving infected and uninfected cell DNA with *Sal*I in combination with *Xho*I. Each of these enzymes cleave unmethylated M-MuLV DNA at a single site (see Fig. 1). Combined cleavage would release a 2.2-kb fragment only from integrated M-MuLV proviruses unmethylated at both sites. For standardization, infected and uninfected cell DNAs were also cleaved with *Bam*HI, which cuts a DNA sequence that does not contain CpG. Cleavage of infected cell DNA with *Bam*HI releases a 3.0-kb M-MuLV-specific fragment (1), and the amount of this fragment generated (indicated by the intensity of hybridization) represents an internal *Bam*HI fragment released from each proviral integration.

A9 and NIH-3T3 DNAs were digested with *Sal*I plus *Xho*I, and equal amounts were digested with *Bam*HI (Fig. 3A). After digestion, the DNA fragments were analyzed by gel electrophoresis and hybridization in situ to the gel with

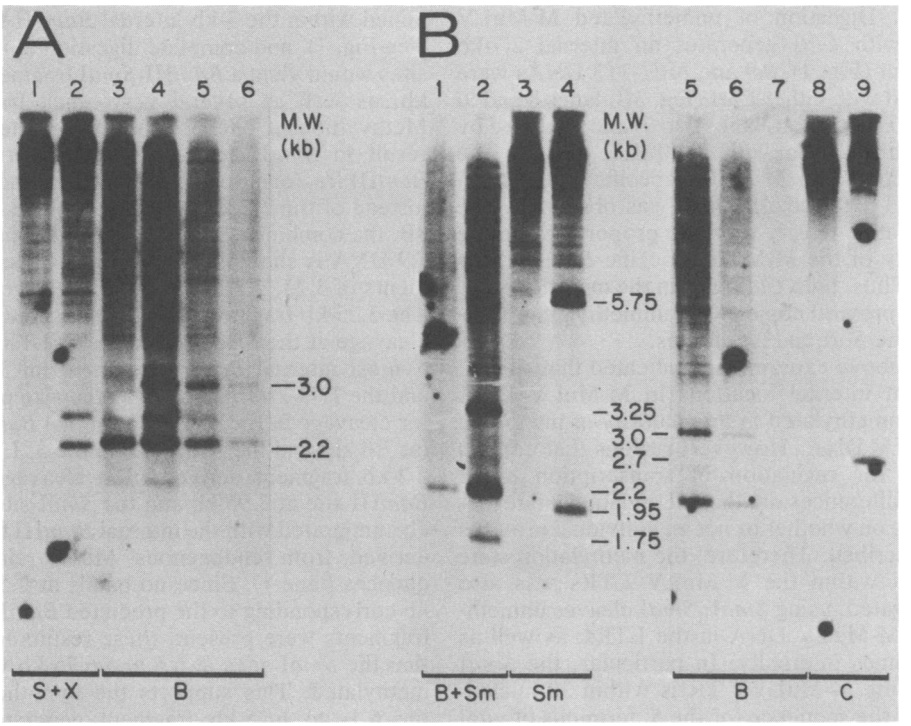


FIG. 3. Methylation at *Sal*I, *Xho*I, *Cla*I, and *Sma*I sites in proviral DNA. NIH-3T3 and A9 DNAs were digested with various enzymes or combinations of them (3 μg per digest). The digested DNA was resolved by electrophoresis in a 0.8% agarose gel and hybridized in situ with M-MuLV cDNA (0.5×10^6 cpm/ml). An autoradiogram of the washed gel is shown (4-day exposure). (A) Lanes 1 and 3 contain NIH-3T3 DNA; all others contain A9 DNA. Lanes 1 and 2, Digestion with *Sal*I and *Xho*I; lanes 3 and 4, digestion with *Bam*HI; lanes 5 and 6, digestion of smaller amounts of A9 DNA with *Bam*HI (1 and 0.3 μg, respectively). (B) Lanes 1, 3, and 8, Digestion of NIH-3T3 DNA with *Bam*HI plus *Sma*I (lane 1), with *Sma*I (lane 3), or with *Cla*I (lane 8); lanes 2, 4, 5, 6, 7, and 9, digestion of A9 DNA with *Bam*HI plus *Sma*I (lane 2), *Sma*I (lane 4), *Bam*HI (3, 1, and 0.3 μg of A9 DNA in lanes 5, 6, and 7, respectively), or *Cla*I (lane 9). Digestion of both infected and uninfected cell DNA with *Bam*HI yielded a hybridizing fragment of 2.3 kb. This fragment represented an internal fragment derived from multiple copies of endogenous MuLV-related sequences present in all mouse strains (11; R. Mural, personal communication). M.W., Molecular weight.

radioactive M-MuLV cDNA. Hybridization to gels in situ was approximately five times as efficient as hybridization to blot transfers when M-MuLV cDNA was used, and one-fifth to one-tenth as much DNA as for blot transfers (3 to 6 μg of cell DNA per lane) was loaded onto the agarose gels. A 2.2-kb M-MuLV-specific *Sal*I-plus-*Xho*I fragment was released from M-MuLV clone A9 DNA (lane 2) and not from NIH-3T3 DNA (lane 1), and the intensity of hybridization for this fragment was only slightly less than the larger 3-kb M-MuLV-specific *Bam*HI fragment (lanes 4 to 6). Since the M-MuLV cDNA probe used in these experiments was relatively uniformly representative of the entire M-MuLV genome, a smaller fragment would be expected to hybridize proportionately less cDNA. This therefore suggested that the *Xho*I and *Sal*I sites in most if not all M-MuLV proviral DNA copies were unmethylated.

An M-MuLV-specific fragment of 2.5 kb was also evident in the *Sal*I-*Xho*I double digest of M-MuLV clone A9 DNA (lane 2). The origin of this fragment is presently unclear. It was M-MuLV specific since digestion of NIH-3T3 DNA did not yield it. However, it did not result from partial digestion of M-MuLV proviral DNA, since no other *Sal*I and *Xho*I sites besides those giving rise to the 2.2-kb fragment are present in M-MuLV DNA. The intensity of hybridization was also too great to result from digestion of one M-MuLV provirus which had an *Xho*I or *Sal*I site in adjacent cell DNA at the appropriate distance to the 5′ side of a provirus. One possibility was that the A9 cells were also infected with a variant of MuLV with internal *Sal*I and *Xho*I sites spaced 2.5 kb apart.

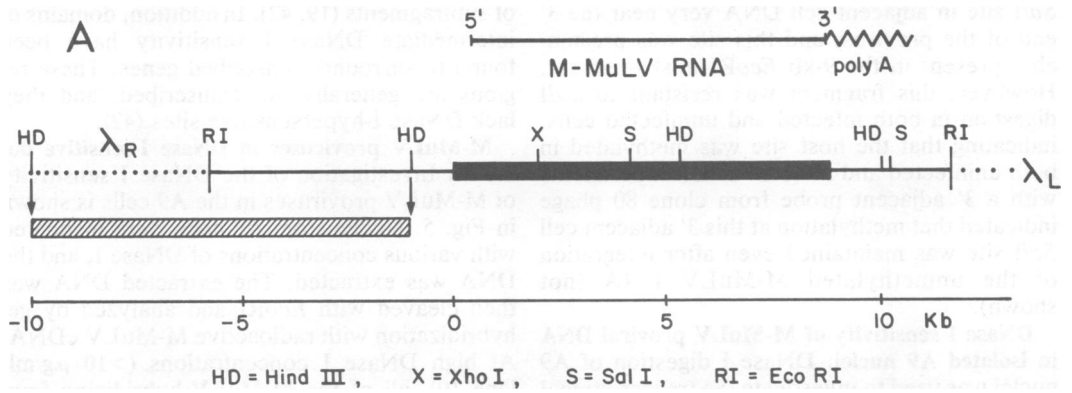
The methylation state of *Cla*I sites in M-MuLV proviral DNA was investigated, using an approach similar to that for the *Sal*I-*Xho*I double

digests. Digestion of unmethylated M-MuLV DNA with *Cla*I generates an internal 2.7-kb fragment (Fig. 1). A9 and NIH-3T3 DNAs were digested with either *Cla*I (Fig. 3B, lanes 8 and 9) or *Bam*HI (Fig. 3B, lanes 5 to 7) and analyzed by gel hybridization with M-MuLV cDNA. The expected 2.7-kb M-MuLV-specific fragment in the *Cla*I-digested A9 DNA was observed (lane 9), and its intensity was also proportional to the intensity of the M-MuLV-specific *Bam*HI fragment. Thus, both *Cla*I sites in the majority of M-MuLV proviral copies were unmethylated, similar to the *Sal*I and *Xho*I sites.

The above experiments indicated that sites at different internal locations in M-MuLV DNA were unmethylated in the majority of integrated M-MuLV DNA. However, regions that contain signals for regulation of transcription might show differences in the methylation state depending on whether or not an individual provirus is transcribed. Therefore, the methylation state of sites within the M-MuLV LTRs was also investigated, using *Sma*I. *Sma*I cleaves unmethylated M-MuLV DNA in the LTRs, as well as three times internally. In particular, the *Sma*I site in the M-MuLV LTR is within 30 nucleotides of the sequence for the 5′ terminus of viral RNA. Digestion of NIH-3T3 and A9 DNA with *Sma*I is shown in lanes 3 and 4 of Fig. 3B. Endogenous MuLV-related DNA from NIH-3T3 cells remained in high-molecular-weight DNA fragments (lane 3). Since several *Sma*I sites have been mapped in the DNA of endogenous MuLV-related organizations (Mural, personal communication), this result indicates that endogenous MuLV-related DNA is methylated. In contrast, the A9 cells yielded major M-MuLV-specific fragments of 5.75 and 1.95 kb, as well as a minor fragment(s) of 6.1 to 6.25 kb (lane 4). Examination of the location of the *Sma*I cleavage sites in M-MuLV DNA suggested that the 5.75-kb fragment resulted from cleavage at the *Sma*I site in the 5′ LTR as well as cleavage at the *Sma*I site at 6.25 kb on the proviral map. Similarly, the 1.95-kb fragment likely resulted from cleavage at the *Sma*I site at 6.75 kb and at the *Sma*I site in the 3′ LTR. An explanation for the minor 6.1- to 6.25-kb fragment could be that the *Sma*I site at 6.25 kb was partially methylated, so that cleavage in the 5′ LTR yielded slightly larger fragments whose 3′ termini resulted from cleavage at the *Sma*I site at 6.6 or 6.75 kb. Alternatively, this fragment might have been generated by *Sma*I digestion of a variant virus. In conclusion, the *Sma*I sites in the LTRs as well as at least some of the internal sites were unmethylated.

To more closely examine the *Sma*I sites, A9 DNA was digested with *Sma*I in combination with *Bam*HI. The internal *Sma*I sites are contained within the 3-kb internal *Bam*HI fragment (see Fig. 1), and complete digestion at the *Sma*I sites would yield a *Bam*HI-*Sma*I fragment of 2.2 kb, as well as several very small fragments. Methylation at one or more *Sma*I sites would result in *Sma*I cleavage of the internal 3-kb *Bam*HI fragment to fragments of 2.5 and 2.65 kb instead of the 2.2-kb fragment. In lane 2 of Fig. 3B, the combined *Bam*HI and *Sma*I digestion of A9 DNA is shown, and M-MuLV-specific fragments of 3.25, 2.2, and 1.75 kb were generated. The 3.25-kb fragment was the size predicted for cleavage at the *Sma*I site in the 5′ LTR and the 5′-most internal *Bam*HI fragment (at 3.75 kb), and the 1.75-kb fragment was the size predicted for cleavage at the 3′-most terminal *Bam*HI site (at 7.0 kb) and the *Sma*I site in the 3′ LTR. The 2.2-kb fragment derived from cleavage at the *Bam*HI site at 3.95 kb and the *Sma*I site at 6.25 kb comigrated with the internal *Bam*HI fragment derived from endogenous MuLV-related sequences (lane 1). Since no bands at 2.5 or 2.65 kb corresponding to the predicted *Bam*HI-*Sma*I fragments were present, these results indicated that the *Sma*I sites at 6.6 and 6.75 kb were not methylated. This supports the hypothesis that the 6.1- to 6.25-kb fragment generated after *Sma*I digestion of A9 DNA was derived from a variant virus. The *Bam*HI-plus-*Sma*I digestion results also supported the conclusion from digestion with *Sma*I alone: that the *Sma*I sites in the LTRs and at internal sites were largely unmethylated.

Methylation of an individual proviral integration. A problem involved with analysis of the A9 cells is that they contain 10 to 15 integrated M-MuLV proviruses, and the observations described above represented the average situation for all of them. Any individual provirus might have had a methylation state different from the average. Therefore, the methylation state of an individual M-MuLV provirus was investigated. For this, a lambda phage recombinant clone carrying a complete integrated M-MuLV provirus was used. The clone used (no. 80) was isolated from the same cloning experiment that we reported previously (3), and its map is shown in Fig. 4A. Whereas the viral sequences in such a recombinant clone would hybridize with all M-MuLV proviruses, single-copy sequences in the adjacent cell DNA in clone 80 would only hybridize to the provirus which was cloned. A *Hin*dIII fragment of clone 80 phage DNA which contained 5′ adjacent cell DNA as well as lambda phage vector DNA (see Fig. 4A) was prepared and radioactively labeled by nick-translation. This probe was then used for hybridization with a gel of A9 and NIH-3T3 cell DNA cleaved with *Eco*RI or *Eco*RI in combination with *Sal*I, *Xho*I, or *Cla*I, similar to Fig. 2 (Fig. 4B). When



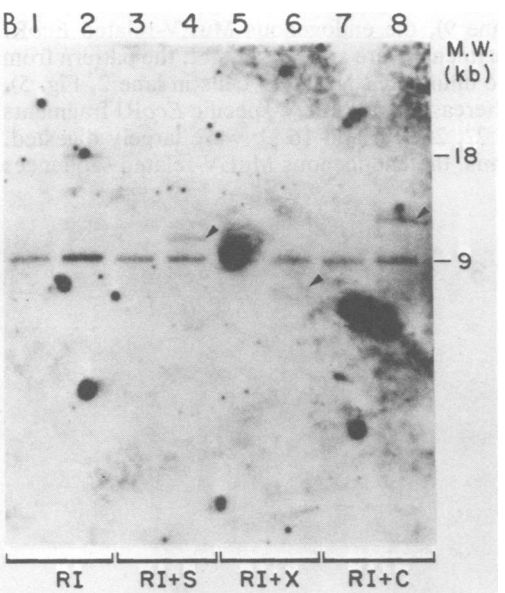


FIG. 4. Methylation of the recombinant clone 80 provirus. (A) A new recombinant lambda phage clone (clone 80) containing an integrated M-MuLV proviral organization from A9 cells was isolated as previously described (3). The clone contained a complete copy of M-MuLV proviral DNA and gave rise to infectious M-MuLV on DNA infection. The provirus was contained in an 18-kb *Eco*RI fragment and contained both 5′ and 3′ adjacent cell sequences. A restriction map of the recombinant phage is shown. H, *Hin*dIII; S, *Sal*I; X, *Xho*I. Preliminary data also indicated at least one site for *Cla*I in the adjacent cell sequences to the 5′ side of the provirus. A *Hin*dIII restriction fragment containing 5′ adjacent cell sequences (as well as sequences from the right lambda cloning arm) was isolated by preparative gel electrophoresis followed by recovery on glass filter paper (6) and was labeled by nick-translation. The location of this fragment is shown in the shaded bar. Poly A, Polyadenylate. (B) NIH-3T3 (odd-numbered lanes) and A9 (even-numbered lanes) DNAs (3 μg per digest) were digested with *Eco*RI or *Eco*RI in combination with *Sal*I, *Xho*I, or *Cla*I. The digests were resolved by electrophoresis in a 0.6% agarose gel and hybridized in situ with the nick-translated 5′ adjacent cell probe of (A) (10^6 cpm/ml). Exposure was for 15 days. Nonspecific binding of labeled DNA to the gel was generally a greater problem when nick-translated probes were used instead of M-MuLV cDNA. M.W., Molecular weight.

the DNAs were digested with *Eco*RI alone, both NIH-3T3 and A9 DNAs showed a 9-kb fragment, and the A9 DNA showed a less abundant fragment of 18 kb (lanes 1 and 2). The A9 cell 18-kb fragment was the same size as the *Eco*RI fragment containing the proviral DNA inserted into clone 80 and represented the provirus from which it was cloned. The 9-kb fragment present in both uninfected and infected cells represented the alleles in host cell DNA which did not receive an M-MuLV integration (the host site) and, as expected, was approximately 8.8 kb smaller than the fragment into which an integration occurred. The greater intensity of the 9-kb fragment than the 18-kb fragment in the A9 cells likely reflected the fact that the parental NIH-3T3 cells are approximately tetraploid (with four copies of the host site), and M-MuLV DNA integration only occurred in one of them.

Digestion of the infected and uninfected cell DNAs with *Eco*RI in combination with *Sal*I, *Xho*I, and *Cla*I reduced the size of the 18-kb A9-specific fragment to 10 (lane 4), 8.4 (lane 6), and 11 (lane 8) kb, respectively. These sizes were appropriate for cleavage of the clone 80 M-MuLV provirus in the A9 cells at the *Sal*I, *Xho*I, and *Cla*I sites within the M-MuLV DNA. The results indicated that the sites for these enzymes were unmethylated in the clone 80 provirus. Methylation at the more 3′ *Cla*I site could not be tested in this manner, since the probe used was a 5′ adjacent cell probe, and cleavage at the more 5′ *Cla*I was complete.

The map of clone 80 phage DNA revealed a

*Sal*I site in adjacent cell DNA very near the 3′ end of the provirus, and this site was presumably present in the 9-kb *Eco*RI host fragment. However, this fragment was resistant to *Sal*I digestion in both infected and uninfected cells, indicating that the host site was methylated in both uninfected and infected cells. Experiments with a 3′ adjacent probe from clone 80 phage indicated that methylation at this 3′ adjacent cell *Sal*I site was maintained even after integration of the unmethylated M-MuLV DNA (not shown).

DNase I sensitivity of M-MuLV proviral DNA in isolated A9 nuclei. DNase I digestion of A9 nuclei was used to investigate the transcriptional state of M-MuLV proviruses. Weintraub and Groudine (41) showed that digestion of nuclei or chromatin with DNase I preferentially removes transcribed genes. This observation was recently extended, and it was found that sites of DNase I hypersensitivity are generally located at the 5′ ends of transcribed genes (23, 31, 44). As a consequence, a hypersensitive DNA restriction fragment is rapidly removed from nuclei digested with DNase I and generates a series of subfragments (19, 42). In addition, domains of intermediate DNase I sensitivity have been found to surround transcribed genes. These regions are generally not transcribed, and they lack DNase I-hypersensitive sites (42).

M-MuLV proviruses in DNase I-sensitive domains. Investigation of the DNase I sensitivity of M-MuLV proviruses in the A9 cells is shown in Fig. 5. Nuclei from A9 cells were incubated with various concentrations of DNase I, and the DNA was extracted. The extracted DNA was then cleaved with *Eco*RI and analyzed by gel hybridization with radioactive M-MuLV cDNA. At high DNase I concentrations (>10 μg/ml; lane 10), all of the M-MuLV-hybridizing fragments were digested. However, at intermediate concentrations, differential sensitivity of M-MuLV-hybridizing fragments was observed. In A9 nuclei digested with 5 μg of DNase I per ml (lane 9), the endogenous MuLV-related *Eco*RI fragments were still present (cf. the pattern from the uninfected NIH-3T3 cells in lane 2, Fig. 5), whereas the M-MuLV-specific *Eco*RI fragments of 22, 20, 18, and 16 kb were largely digested. Thus, the endogenous MuLV-related sequences

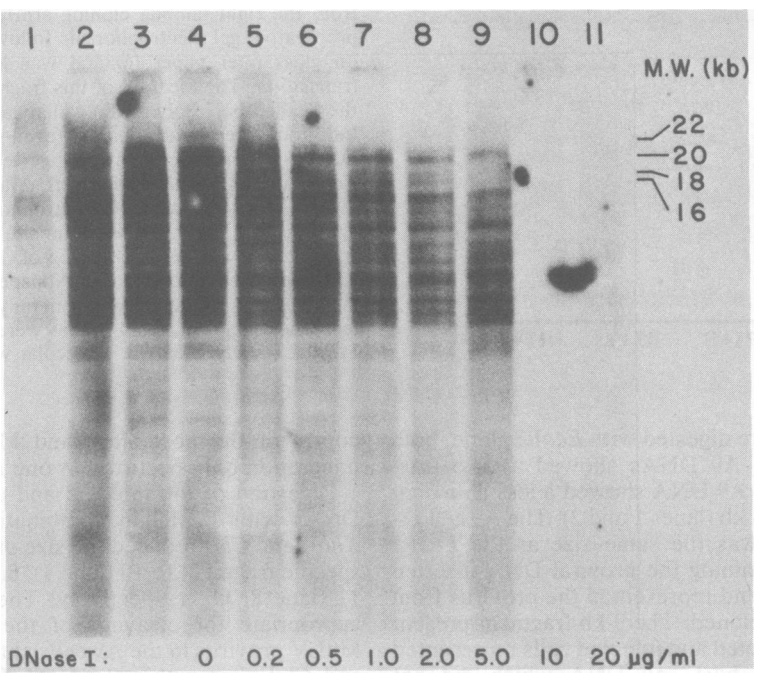


FIG. 5. DNase I sensitivity of *Eco*RI fragments containing M-MuLV proviral DNA. Nuclei from A9 cells were isolated and incubated for 20 min at 15°C with different concentrations of DNase I, and the DNA was then extracted. For controls, DNA was extracted from NIH-3T3 and A9 nuclei that had not been incubated at all. A 6-μg portion of DNA from each sample was digested with *Eco*RI and analyzed by gel hybridization with M-MuLV cDNA as in the legend to Fig. 3. Exposure was for 4 days. Lanes 1 and 2, Two different preparations of NIH-3T3 DNA; lane 3, control A9 DNA (extracted directly). Lanes 4 to 11, DNA from A9 nuclei incubated with the following amounts of DNase I: 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10, and 20 μg/ml, respectively. Essentially all high-molecular-weight (M.W.) DNA was digested in the last two samples.

(which are not transcribed in NIH-3T3 cells; 4) are in the DNase I-resistant configuration, whereas the M-MuLV proviral integrations are in DNase I-sensitive domains. However, due to the large number of M-MuLV-hybridizing fragments in the *Eco*RI pattern, it was not possible to distinguish whether individual integrations contained DNase I-hypersensitive sites or not. Therefore, individual integrations were examined.

DNase I sensitivity of individual integrations. In the first approach to examining the DNase I sensitivity of individual M-MuLV integrations, the same DNA samples from the DNase I-digested A9 nuclei used in Fig. 5 were digested with *Cla*I instead of *Eco*RI and analyzed by electrophoresis and gel hybridization (Fig. 6). The majority of M-MuLV-hybridizing DNA was very high molecular weight and reflected the fact that most of the *Cla*I sites in cellular DNA are methylated and resistant to cleavage. The internal 2.7-kb *Cla*I fragment released from all of the M-MuLV proviruses was also evident, as expected. In addition, two M-MuLV-specific fragments of 7 and 5.8 kb were present in undigested A9 nuclear DNA (lane 2). These fragments presumably represented the 5′ portions of two M-MuLV proviruses which also contained unmethylated *Cla*I sites in the 5′ adjacent cell DNA. This conclusion was supported by the fact that a nick-translated fragment from the 5′ portion of cloned unintegrated M-MuLV DNA (the *Ava*I fragment from 2.6 to 3.8 kb on the proviral map) hybridized with both fragments (not shown). Both of these fragments were absent in the DNA samples which had been digested with 1-μg/ml (lane 6) or higher concentrations of DNase I. A larger fragment (9.5 kb) also present in uninfected cells, and thus containing endogenous MuLV-related DNA, was not digested by 5 μg of DNase I per ml. The 5.8- and 7-kb *Cla*I fragments were more DNase I sensitive than were the M-MuLV-specific *Eco*RI fragments from the same DNA samples. For instance, in Fig. 5, the 18- and 16-kb M-MuLV-specific *Eco*RI fragments could still be detected in DNA from nuclei digested with 2 μg of DNase I per ml. These results suggested that the 5.8- and 7-kb M-MuLV-specific *Cla*I fragments contained DNase I-hypersensitive sites. In fact, samples from the higher DNase I digestion concentrations also showed subfragments for these proviral integrations (barely visible in Fig. 6, but more visible in autoradiograms which had not been preflashed before exposure of the film). The *Cla*I digestions thus also served to identify the DNase I concentrations in which hypersensitive sites were detected in this particular DNase I digestion. These results indicated that many of the M-MuLV proviruses visible in Fig. 5 lack

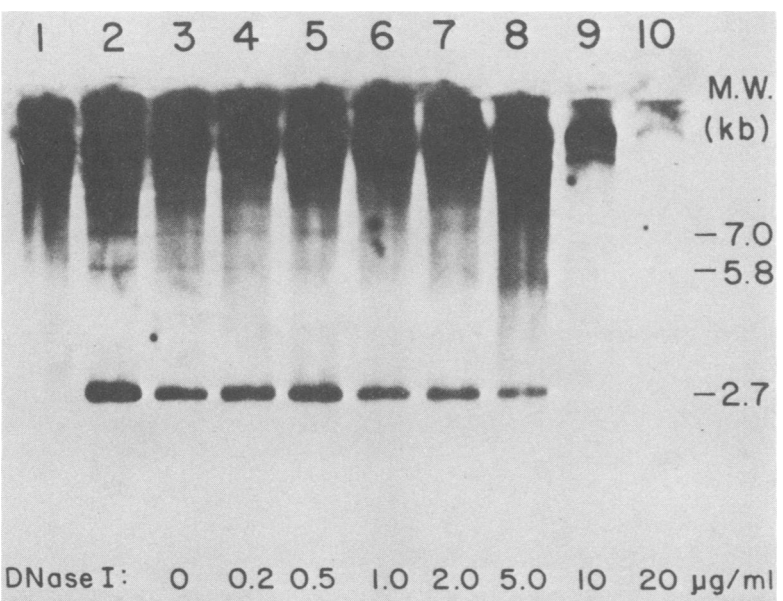


FIG. 6. *Cla*I digestion of DNA from DNase I-digested A9 nuclei. The same DNA samples described in the legend to Fig. 5 were digested with *Cla*I instead of *Eco*RI (6 μg per digest) and analyzed by gel hybridization with M-MuLV cDNA. Exposure was for 10 days. Lane 1, NIH-3T3 control DNA; lane 2, unincubated A9 control DNA; lanes 3 to 10, DNA from the A9 nuclei incubated with 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10, and 20 μg of DNase I per ml, respectively. M.W., Molecular weight.

hypersensitive sites and were thus in regions of intermediate DNase I sensitivity.

A second approach to examining the DNase I sensitivity of an individual provirus was to use the 5′ adjacent cell probe described in Fig. 4 for the clone 80 proviral integration in A9 cells. In Fig. 7 the same DNA samples as in Fig. 5 and 6 were digested with *Eco*RI and analyzed by electrophoresis and gelhybridization with nick-translated clone 80 5′ adjacent cell probe. As in Fig. 4B, the integration at 18 kb and the host site at 9 kb were evident in undigested A9 DNA. Examination of the DNase I-digested DNA samples indicated that the clone 80 integration did not contain hypersensitive sites, since it was still detectable in the DNA sample which had been digested with 2 μg of DNase I per ml. This integration therefore appeared to be in a domain of intermediate DNase I sensitivity. Also indicated was that the 9-kb fragment corresponding to the clone 80 host site was in DNase I-resistant chromatin.

DISCUSSION

In the results reported here the methylation state and DNase I sensitivity of chromatin containing M-MuLV proviral DNA and endogenous MuLV-related DNA were tested in productively infected mouse cells. The methylation state was examined at nine sites distributed throughout the M-MuLV genome. In a cell line which contained 10 to 15 copies of integrated M-MuLV DNA, most if not all of the proviruses were not detectably methylated at these sites, whereas endogenous MuLV-related DNA was methylated.

Chromatin containing M-MuLV proviruses or endogenous MuLV-related DNA showed three different types of DNase I sensitivity in these experiments. Endogenous MuLV-related sequences were in the DNase I-resistant configuration, consistent with their nontranscribed state in both infected and uninfected NIH-3T3 cells. In contrast, the integrated M-MuLV proviruses were in domains of greater DNase I sensitivity, since moderate digestion preferentially removed them in comparison to the endogenous MuLV-related sequences. Two individual M-MuLV proviruses were found to be even more sensitive to DNase I digestion than the bulk of the M-MuLV proviruses and may have contained DNase I-hypersensitive sites. These three classes of DNase I sensitivity (resistance, intermediate sensitivity, and hypersensitivity) resemble those observed by Weintraub and co-workers

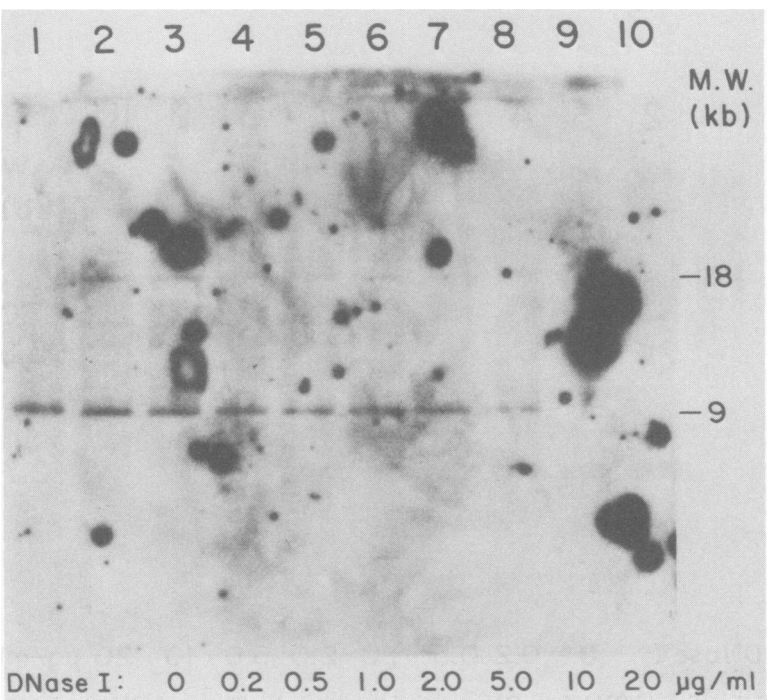


FIG. 7. DNase I sensitivity of the clone 80 proviral integration. The same DNA samples described in the legend to Fig. 5 were digested with *Eco*RI and analyzed by gel hybridization, using the nick-translated recombinant clone 80 adjacent cell probe described in the legend to Fig. 4. Number of the lanes is the same as in the legend to Fig. 6. Exposure was for 18 days. M.W., Molecular weight.

for chromatin containing the globin genes of chick cells (31, 42), and analogy with their experiments suggests that only the A9 cell M-MuLV proviral integrations with hypersensitive sites might be transcribed. The suggestion that only M-MuLV proviral integrations with DNase I hypersensitive sites are transcribed awaits direct test, perhaps by the technique of in vitro nuclear runoff transcription (42). Previous experiments suggested that only a minority of integrated M-MuLV proviruses were expressed in productively infected fibroblasts (5, 13).

If the correlation between transcriptional activity and DNase I sensitivity proves to be correct for this system, these results suggest that hypomethylation of exogenously infecting M-MuLV proviral DNA is not correlated with transcriptional activity. Other factors may be regulating transcription in these cells.

The putative lack of transcription of a majority of the M-MuLV proviruses in the A9 cells was also not due to a defect in the proviral DNA. Six M-MuLV proviral organizations have been isolated by recombinant DNA techniques from the A9 cells, at least one of which (clone 80) lacks DNase I-hypersensitive sites. Nevertheless, all of these give rise to infectious M-MuLV upon DNA infection into NIH-3T3 cells at high efficiency (3; H. Fan and Y. C. Lin, unpublished data).

A general correlation between transcription and hypomethylation has been documented for both retroviral and host cellular genes (7, 10, 19, 32, 42). In the case of retroviral genes, endogenously transmitted retroviral copies which are transcriptionally inactive have been found to be highly methylated (7, 28, 38; see Fig. 4). When endogenous viral copies are expressed (sometimes as the result of reinfection), the proviral DNA is hypomethylated. Furthermore, transcriptional activation of avian endogenous retroviruses (19), as well as the mouse metallothionine gene (8), has been achieved by treatment of cells with 5-azacytidine, an agent which blocks DNA methylation. However, the experiments reported here differ from those of van der Putten et al. (38) and Groudine et al. (19) in that they were performed on cells exogenously infected with M-MuLV. M-MuLV DNA synthesized during exogenous infection is unmethylated, as shown by the fact that it can be cleaved by methyl-sensitive restriction enzymes (17, 39). In eucaryotic cells the DNA methylation pattern is propagated during replication (24, 43). It is thus not surprising that the unmethylated M-MuLV DNA remained unmethylated during integration into the host chromosomal DNA. Rather, it is surprising that some exogenously acquired M-MuLV proviral copies may be transcriptionally inactive. The results reported here suggest that whereas hypomethylation of DNA may be necessary for transcriptional activity, hypomethylation alone is not sufficient to cause it.

Another possible control for the transcriptional activity of exogenously acquired M-MuLV proviruses is the transcriptional state or nature of the surrounding host chromatin. In particular, proviral DNA might be expressed when integrated into active (DNase I hypersensitive, hypomethylated) host chromatin, but not expressed if integrated into inactive chromatin. Such a situation has been suggested for integrated proviral copies of murine mammary tumor virus integrated into rat HTC cells (14a). It will be possible to test this when a lambda recombinant clone from the M-MuLV clone A9 cells containing a proviral DNA which is transcribed (DNase I hypersensitive) is identified. In fact, evidence consistent with this hypothesis was found in the results reported here. For the clone 80 integration (lacking DNase I-hypersensitive sites), the methylation state and DNase I sensitivity of the adjacent cell DNA as well as in the host site without integrated proviral DNA could be deduced. The host site was resistant to DNase I digestion (Fig. 7), and a *Sal*I site in the host DNA near the integration remained methylated (Fig. 4B). In the case of the two DNase I-hypersensitive M-MuLV integrations identified in Fig. 6, both contained unmethylated *Cla*I sites in the 5′ adjacent cell DNA relatively close to the integrated provirus.

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