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Moloney murine leukemia virus-induced tumors: recombinant proviruses in active chromatin regions

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ABSTRACT

The DNase I sensitivity of chromosomal DNA regions carrying integrated proviral genomes of Moloney (M-MuLV) and AKR Murine Leukemia Virus (AKR-MuLV), and the cellular homologue of the mos-gene (c-mos) of Moloney Sarcoma Virus (MSV) were studied in tumor tissues of leukemic mice. The genetically transmitted sequences of M-MuLV, AKR-MuLV, and the c-mos gene are all in DNase I resistant chromatin conformations in M-MuLV-induced tumors. Each M-MuLV-induced tumor contained at least one somatically acquired integrated recombinant MuLV genome that displayed two main characteristic features of active chromatin: a) a configuration hypersensitive to DNase I, and b) extensive hypomethylation. DNase I hypersensitive sites were mapped at the junction of cellular sequences and the 5'-viral large terminal repeat (LTR). Expression of a recombinant MuLV seems therefore to be a necessary feature to maintain the transformed state.

INTRODUCTION

Moloney (M-MuLV) and AKR (AKR-MuLV) murine leukemia viruses can induce leukemogenesis in a variety of mouse strains. Leukemogenesis is accompanied by somatic amplification and reintegration of M-MuLV (1-5) and AKR-MuLV (6-10) sequences respectively in new chromosomal sites of tumor tissues.

M-MuLV and AKR-MuLV induce predominantly thymus-dependent leukemias in appropriate hosts. Leukemia development follows the expression of high titers of the virus in the blood (2,11). The thymus-dependent leukemia of viral (M-MuLV) etiology in Balb/Mo mice (12) is probably due to the transient expression of the endogenous M-MuLV genome (Mov-1; 1,4,5) on chromosome number 6 (13), which leads to infection of the lymphatic target tissues where virus-specific RNA can be detected (3) concomitantly with proviral DNA amplification (1,3-5).

Although Balb/Mo, Balb/c and AKR mice carry AKR-MuLV as an endogenous provirus (4,8-10), the expression of this virus is seen occasionally later in life of Balb/c mice (14). Apparently, the expression of the AKR endogenous provirus is under strong host control in these mice in which the Fv-1 locus could well play a major role (15,16).

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Previous studies have shown characteristic features of M-MuLV (1,4,5) and AKR-MuLV (7-10) induced leukemias. Outgrown tumors, largely composed of one or a few clonal cell populations, harbor recombinant proviral genomes integrated in multiple unique sites of chromosomal DNA. The recombinant proviral DNAs are all env-gene recombinants, which have acquired cellular sequences in the 3'-portion of the viral genome comprizing information for the amino-terminal part of the env-glycoprotein. Every lymphoma induced by M-MuLV or AKR-MuLV harbors this same type of recombinant provirus, although the copy number varies in different tumors (2,5). This would favor a model which defines a recombinant provirus as a prerequisite for the onset and maintenance of the transformed state. No common integration sites, providing a promoter for neighbouring cellular sequences (as observed for ALV in ALV-induced tumors in birds; 17-19) have been detected so far.

If the expression of a recombinant proviral genome is required for the maintenance of the transformed state then at least some of the reintegrated genomes are expected to be localized in active chromatin regions. Several DNase I probing studies of chromatin have provided convincing evidence that "active" chromatin has an altered, more accessible configuration (20,21; for review see 22). The sensitivity to DNase I has been correlated with the commitment or competence to synthesize a given gene product (21,23). Furthermore, chromosomal domains active in transcription have been identified as contiguous chromosomal structures exhibiting high and moderate DNase I sensitivity (20,21). DNase I hypersensitive sites have been detected almost exclusively at the 5'-ends of potentially active genes (21,23-26) and probably reflect exposed DNA sequences which are essential for gene activation (23,27). We have analyzed the preferential sensitivity of viral genomes for DNase I. It appears that chromatin comprizing proviral genomes in outgrown tumors can be devided in three different DNase I sensitivity classes i.e. in configurations resistant, moderately sensitive and hypersensitive to DNase I. Two molecular features characteristic for active DNA-sequences could be assigned to part of the recombinant MuLV genomes integrated in tumor-tissue DNA: 1) part of the recombinant MuLV genomes in tumors display a configuration hypersensitive to DNase I, 2) all somatically acquired MuLV sequences are hypomethylated in contrast to the genetically transmitted copy Mov-1 in tumors from Balb/Mo mice. These characteristic features of recombinant MuLV genomes in DNA from tumor tissues strongly supports the hypothesis that the expression of a recombinant MuLV encoded gene product is essential for the maintenance of transformation.

MATERIALS AND METHODS

Mice, cell lines, viruses, cDNA probes and hybridizations

Mice, cell lines and isolation procedures for M-MuLV clone 1A and AKR-MuLV have been described previously (4). The injection of newborn Balb/c mice with M-MuLV clone 1A was performed intraperitoneally (2,4,5).

The selection of M-MuLV and AKR-MuLV specific cDNA probes, the preparation and characteristics of the M-MuLV specific plasmid probes and hybridization procedures have also been outlined previously (4,5,8).

Preparation of cell nuclei

Nuclei were isolated by a procedure modified from those described before (20,28), in order to reduce the activity of endogenous DNases. All manipulations were carried out at 0 to 4 °C. Either frozen (-80 °C) or fresh mouse tissues (0.2-0.8 g) were homogenized in 10 ml PAP IV buffer (17.1 % sucrose; 15 mM Tris-HCl, pH 7.5; 0.5 mM spermidine; 0.15 mM spermine; 1 mM EDTA; 1 mM EGTA; 25 mM KCl) by douncing 10x in a dounce homogenizer (Pistol A); 5 ml PAP IV + 0.2 % NP-40 were added in two portions and dounced 1x and 2x respectively; the homogenate was filtered through cheese cloth and centrifuged 5 min at 3000 x g; the nuclei were washed twice with PAP IV buffer without NP-40 and resuspended in 5 ml PAP IV buffer; the suspension was filtered through scrynell nylon and the E260 was determined by sampling into 1 % SDS, 0.2 M NaOH. Nuclei were pelleted once more, washed in 5 ml RSB_5 buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaCL; 5 mM $MgCl_2$) and finally resuspended in RSB_5 buffer at a DNA concentration of 1 mg/ml. EGTA was added to a final concentration of 0.2 mM. Exogenous DNase I (Sigma) digestions were carried out for 2 min at 25 °C in Eppendorf tubes containing 1.4 mM $CaCl_2$ and 0.3-5 µg/ml DNase I (1505Kunitz/mg protein). DNase I storage buffer contained: DNase I, 1 mg/ml; 0.15 M NaCl; 1 mg/ml BSA. Reactions were terminated by adding 1 volume 1 % SDS; 5 mM EDTA; 20 mM Tris-HCl, pH 8.0, and incubated for 1 min at 60 °C. DNA was purified by Proteinase K (50-100 mg/ml) treatment, subsequent extractions with phenol and chloroform and two precipitations with ethanol.

Restriction analyses

Restriction analyses of high molecular weight DNAs from tissues were carried out as described previously (4,5,8). Elution of DNA fragments from agarose gel slices was performed either by electroelution (4,8) or by adsorption to glass beads (29). Marker DNA fragments for size determination as shown in Fig. 1, 3, and 4 represent Hind III fragments of phage lambda DNA.

RESULTS

Chromatin conformation of Mov-1, Akv, c-mos, and somatically acquired MuLVs

Previous studies revealed an increase in M-MuLV specific RNA sequences, concomitantly with proviral DNA amplification, in target tissues of Balb/Mo and newborn infected Balb/c mice (2,3). Although it has been reported that the majority of the new somatically acquired sequences in tumors are preferentially digestible by DNase I (28), these studies did not indicate whether a difference in sensitivity to DNase I existed between the individual reintegrated and genetically transmitted viral copies. Furthermore, it remained unclear whether DNase I sensitivity is confined to specific proviral copies (authentic or recombinant MuLV) or to specific sequences of proviral DNA. Therefore, the DNase I sensitivity of chromosomal DNA regions, carrying integrated proviral genomes of authentic and recombinant M-MuLVs were studied in tumor tissues of leukemic mice. The DNase I sensitivity of two cellular sequences, the AKR-MuLV proviral genome (4,8) and the cellular homologue of the mos-gene of MSV (c-mos) (30), were taken for reference.

Nuclei were isolated from both tumor and non-tumor tissues of individual mice (both Balb/Mo and Balb/c). The differential sensitivity of the individual proviral M-MuLV genomes for DNase I was determined after incubating nuclei with different concentrations of DNase I; DNA was extracted, digested with Eco RI (which does not cleave the authentic M-MuLV proviral DNA; 4,5), DNA fragments separated on size and after Southern transfer hybridized to a M-MuLV specific cDNA probe (Fig. 1). This probe was prepared as described previously (5) and detects only M-MuLV sequences while other endogenous viral sequences in Balb/Mo and Balb/c mice are not recognized. Furthermore, the M-MuLV specific probe displays hybridization with genomic fragments derived from different locations of cloned M-MuLV proviral DNA. Therefore, the M-MuLV specific probe can be used to detect M-MuLV subgenomic fragments in cellular DNA. The results show that some of the reintegrated MuLV sequences are hypersensitive to DNase I (closed arrows). The Mov-1 M-MuLV proviral locus in both non-tumor (brain) and tumor tissues is not sensitive to DNase I. Even at considerably heigher DNase I concentrations (2.5-5 µg/ml) the Mov-1 M-MuLV DNA was relatively resistant whereas the somatically acquired MuLV copies were all more sensitive. When naked DNA was probed with DNase I both the Mov-1 M-MuLV DNA and the reintegrated MuLV genomes were not preferentially digested with DNase I (data not shown). These analyses show that the different MuLV sequences in M-MuLV-induced tumors (several tumors from both Balb/Mo and Balb/c mice, infected when newborn with M-MuLV have been tested) show a differential sensitivity to DNase

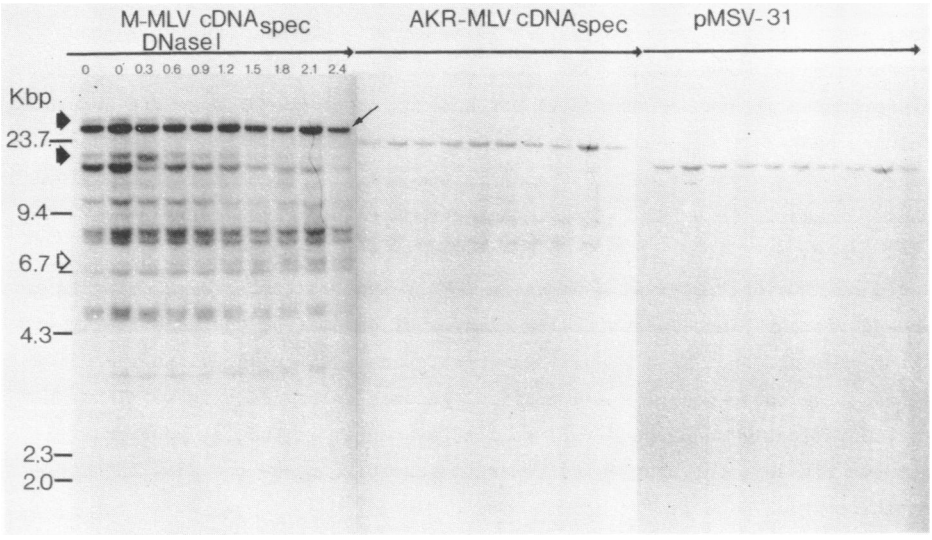

Fig. 1. Sensitivity to DNase I of Mov-1, Akv, c-mos, and somatically acquired MuLVs in M-MuLV-induced tumor-tissue chromatin. Nuclei were isolated from tumor tissue from a Balb/Mo mouse (M9) as described in Materials and Methods and incubated with different concentrations of DNase I as indicated (0-2.4 µg/ml). DNA from each sample was subsequently purified, 10 µg restricted with Eco RI and screened with different probes after Southern blotting. M-MuLV and AKR-MuLV specific probes were prepared as described previously (5,8). For detection of cellular mos-sequences (c-mos) a nick-translated probe of pMSV-31 was used (30). The large arrows indicate reintegrated MuLV sequences hypersensitive to DNase I. The fragment indicated by a small arrow indicates the genetically transmitted Mov-1 M-MuLV DNA in Balb/Mo mice. The fragment, newly emerging after DNase I digestion and subsequent cleavage by Eco RI, is indicated by an open arrow.

I. All reintegrated genomes are more sensitive to DNase I than the genetically transmitted genome. In order to relate the DNase I sensitivity of the Mov-1 M-MuLV DNA and reintegrated MuLV DNAs with other proviral and cellular genes the blots shown in Fig. 1 were hybridized with an AKR-MuLV specific cDNA probe (8) and a mos-specific plasmid probe (30). The single genetically transmitted ecotropic AKR proviral DNA (in a 20 kbp Eco RI DNA fragment (8)) appeared in a DNase I resistant chromatin conformation in both tumor and non-tumor (not shown) tissues from Balb/Mo and Balb/c mice. Other endogenous viral sequences can cross-react with AKR-MuLV cDNA probe using hybridization conditions allowing some base-pair mismatching (8) (Fig. 1, panel 2). These cross-reacting endogenous proviral sequences also appeared rather resistant to DNase I. In addition we probed the DNase I sensitivity of the cellular homologue of MSV speci-

fic mos-sequences as an internal reference of a cellular non-viral gene. These sequences were previously identified in a 14 kbp Eco RI DNA fragment from uninfected mouse cells (30). The cloned cellular DNA fragment contains an uninterrupted stretch of about 1.1 kbp homologous to Mo-MSV specific sequences; a clone, pMSV-31 (30), containing this mos-specific DNA fragment was used as a probe. These analyses revealed that also the cellular homologue of the viral mos-sequences is in a DNase I resistant chromatin conformation in M-MuLV-induced tumors.

In conclusion, proviral genomes in M-MuLV-induced tumors were detected in three different DNase I sensitivity classes of chromatin; the genetically transmitted AKR-MuLV and Mov-1 M-MuLV DNA were found in DNase I resistant configurations like the c-mos gene and proviral genomes cross-reacting with AKR-MuLV cDNA. Somatically acquired M-MuLV related sequences in tumors, however, fall into two classes, one displaying moderate sensitivity and one showing hypersensitivity to DNase I.

Location of DNase I sensitive sites with respect to proviral genomes

Previously it was established that M-MuLV-induced tumors contain both authentic and recombinant MuLV proviral sequences that display a general structure as shown in Figure 2 (5). The recombinant MuLV proviral DNAs display some very characteristic restriction sites not present in authentic M-MuLV proviral DNA: an Eco RI site at 6.9 kbp and a Bam HI site at 6.3 kbp.
To determine whether fragments hypersensitive to DNase I comprize 3'-ends of recombinant MuLV genomes or sequence information for authentic M-MuLV and/or a 5'-part of a recombinant MuLV, Eco RI DNA fragments of the tumor M9 (Fig. 1) were hybridized to three M-MuLV-specific plasmid probes (5). These probes recognize different parts of the M-MuLV genome (Fig. 2), and are capable of recognizing M-MuLV-specific sequences among the background of endogenous viral genomes (5). Fig. 3 shows that fragments that are hypersensitive to DNase I all hybridize to MS-2, MS-3, and MS-4.
This indicates that these fragments contain either an authentic M-MuLV genome and/or a 5'-part of a recombinant MuLV DNA (up to the Eco RI site at 6.9 kbp). Furthermore, the intensities of hybridization of some Eco RI DNA fragments indicates the presence of at least two proviral copies among such fractions. Additional restriction analyses (see last section) revealed that the two DNase I hypersensitive Eco RI fractions (Fig. 1) contain only a 5'-end of a recombinant MuLV provirus. None of the fragments which only hybridized to MS-2 and therefore contain 3'-parts of reintegrated recombinant proviruses were hyper-

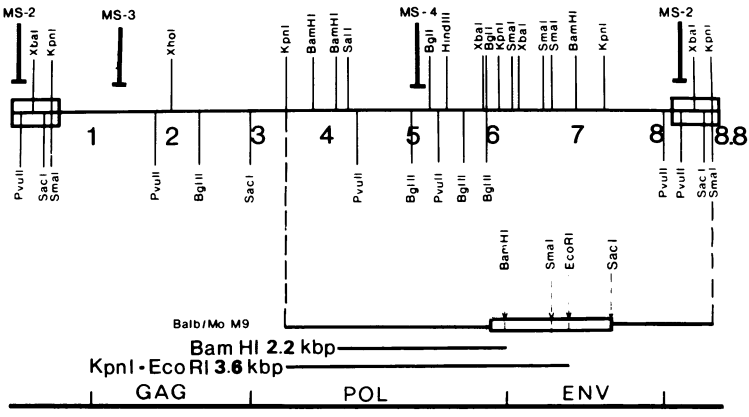

Fig. 2. Recombinant MuLV DNA in tumors. The position (in kbp) of some relevant restriction sites are indicated on the linear map of authentic (upper line) and recombinant (lower line) M-MuLV proviral DNA. Boxes represent LTR sequences. The restriction map in the middle represents the 5.4 kbp Kpn I DNA fragment which is diagnostic for recombinant MuLV proviral DNA. This map was derived previously for recombinant proviral DNA present in tumor DNA from Balb/Mo mouse M9 (5). The new Bam HI site (at 6.3 kbp) and Eco RI site (at 6.9 kbp) are characteristic for recombinant MuLV DNA, found in every lymphoma (5,8). These new sites are responsible for the detection of tumor-specific Bam HI (2.2 kbp) and Kpn I-Eco RI (3.6 kbp) DNA fragments diagnostic for M-MuLV-induced tumors. In addition, recombinant MuLV DNA in the Balb/Mo tumor M9 carries also a Sac I site at position 7.6 (5). Regions MS-2, MS-3, and MS-4 represent those sequences that are recognized by the respective M-MuLV specific plasmid probes (5).

sensitive to DNase I.

A striking feature of the DNase I analyses of tumor tissue chromatin from both Balb/Mo and Balb/c mice was the appearance of a new fragment of 7.0 kbp after DNase I digestion of nuclei and subsequent cleavage by Eco RI. This fragment was only detected after cleavage with Eco RI and could not be detected after DNase I treatment of chromatin without subsequent restriction or after low-level digestion of pure DNA with DNase I.

To elucidate the structure of this new fragment DNA fractions were isolated after Eco RI digestion of DNA which was isolated from nuclei treated with DNase I at a concentration of 2.1 µg/ml as shown in Fig. 1. Subsequently, DNA from each fraction was cleaved by Bam HI, Pst I, and Sac I (Fig. 4). Lane 8 represents a fraction containing predominantly 7.0 kbp Eco RI DNA fragments. The newly emerging 7.0 kbp DNA fragment appeared to be derived from the 5'-part of one or several reintegrated recombinant proviruses since only restriction fragments diagnostic for recombinant MuLV DNA (Fig. 2) were detected in this

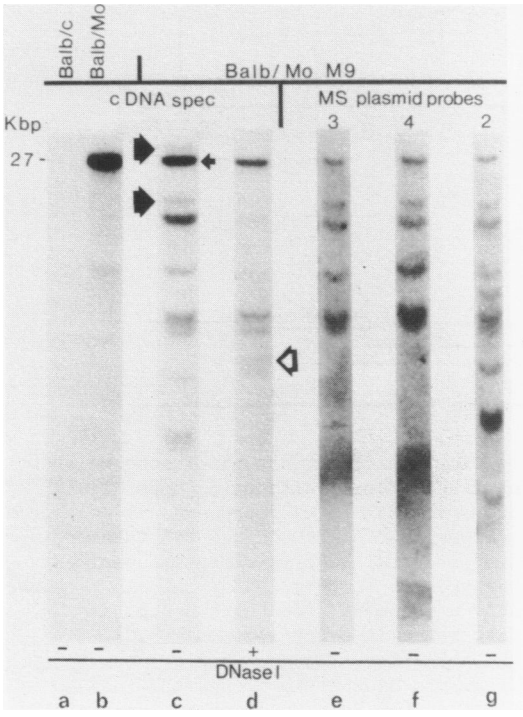

Fig. 3. M-MuLV specific sequences in DNA fragments sensitive to DNase I. Lanes a-g contain 10 µg of Eco RI restricted DNA from Balb/c brain (lane a), Balb/Mo brain (lane b), and Balb/Mo M9 tumor (lanes c-g). Southern blots were hybridized to a M-MuLV specific cDNA probe (lanes a-d) or to M-MuLV specific plasmid probes as indicated (lanes e-g). Lane c contains an Eco RI digest of M9 tumor DNA, whereas lane d contains an Eco RI digest of M9 tumor DNA, isolated from nuclei treated with DNase I (2.1 µg/ml) as shown in Fig. 1. The 27 kbp fragment represents the Mov-1 M-MuLV genome (small arrow). The large arrows indicate the two DNA fragments located in chromatin hypersensitive to DNase I. The newly emerging DNA fragment at 7.0 kbp (lane d) is indicated by an open arrow. The upper DNA fragment, hypersensitive to DNase I, hybridized to MS-2, MS-3, and MS-4 probes (lanes e-g), but was only visible after long exposures (not shown).

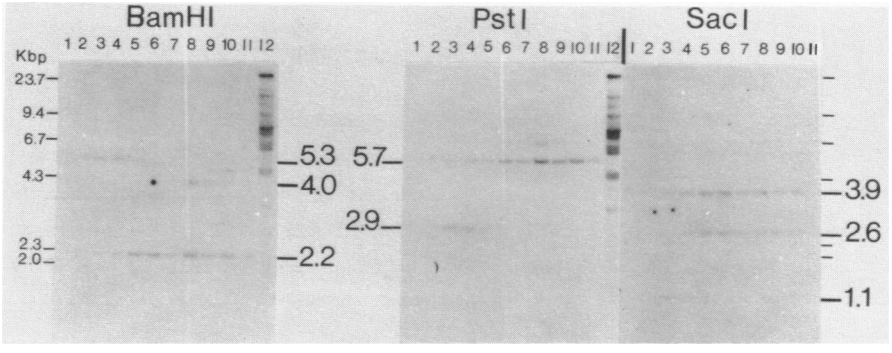

Fig. 4. M-MuLV sequences in the newly emerging 7.0 kbp fragment. 300 µg DNA, isolated from nuclei (Balb/Mo tumor M9) treated with 2.1 µg/ml DNase I (Fig. 1), was restricted with Eco RI, fragments separated on a 0.6 % agarose gel and the gel part containing the 6.5-7.5 kbp Eco RI DNA fractions was sliced into 11 fractions (lane 1= 6.5 kbp; lane 11= 7.5 kbp): each fraction was subsequently analyzed by further restriction with Bam HI, Pst I, and Sac I respectively, and Southern blots were screened with a M-MuLV specific cDNA probe. Lanes 12 contain an Eco RI digest of tumor M9 DNA, isolated from DNase I treated (2.1 µg/ml) nuclei. Large numbers indicate the length of fragments hybridizing to the M-MuLV specific cDNA probe in each gel-slice.

new fragment (Fig. 4). Therefore, the specific degradation of some recombinant proviral DNAs in chromatin by DNase I must be ascribed to a DNase I hypersensitive site around the junction of the 5'-LTR and cellular sequences (Fig. 5). The location of this DNase I hypersensitive configuration was further confirmed by hybridizing the Pst I blot (Fig. 4) with MS-2 probe which is complementary to a region at the start of the LTR; in lane 8 a single fragment of 1.1 kbp was detected (not shown). The coding sequences of these recombinant MuLVs, however, are not hypersensitive to DNase I but show moderate sensitivity like most somatically acquired MuLV proviral sequences.

Lane 3 (Fig. 4) represents the fraction containing predominantly 6.7 kbp Eco RI DNA fragments. The 6.7 kbp Eco RI DNA fragment, which hybridizes to the M-MuLV specific cDNA probe, represents a 3'-end of a recombinant MuLV genome as also shown by the differential hybridization characteristics against the MS-plasmid probes (Fig. 3). Cleavage by Bam HI, Pst I, and Sac I (Fig. 4) revealed a restriction map for this 3'-part of a recombinant MuLV (not shown) which revealed that no DNase I hypersensitive site could be mapped in the 3'-part of this recombinant viral DNA and also not in the 4.8 kbp of cellular sequences following the 3'-LTR sequence. We could not find any evidence for other 3'-Eco RI DNA fragments of recombinants hypersensitive to DNase I although such fragments might exist since some Eco RI DNA fractions contain more copies of MuLV proviral sequences. The results suggest that mainly the 5'-ends of some reintegrated recombinant proviruses are in "active" chromatin conformations.

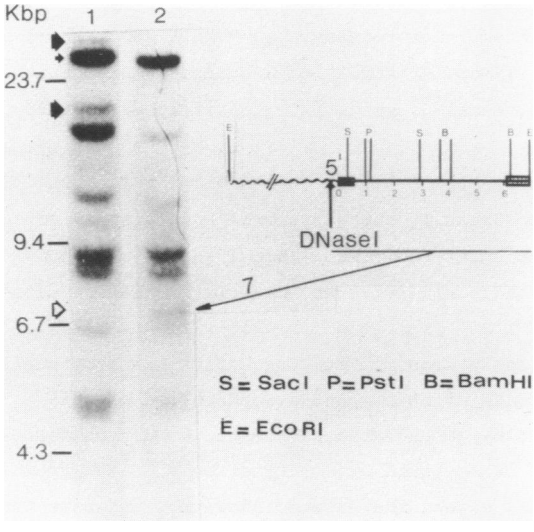

Fig. 5. Restriction map and location of DNase I hypersensitive site on recombinant MuLV proviral DNA. Lanes 1 and 2 show the blot-hybridization results of Eco RI restricted DNA from Balb/Mo tumor M9 without (lane 1) and with (lane 2) prior treatment of nuclei with DNase I. Fragments are marked as in Fig. 1 and 2. The restriction map obtained for the 7.0 kbp fragment is shown; the dashed area indicates the recombined sequences, and the black box represents the 5'-LTR.

DNase I sensitivity and methylation of proviral DNAs in tumors

Differences in methylation patterns of both genetically and somatically acquired M-MuLV sequences have been observed. In tumor tissues the Mov-1 M-MuLV DNA has a different methylation pattern e.g. Sma I and Sal I cleave the Mov-1 M-MuLV DNA from tumor tissues but not from brain (5) and also other tissue specific differences in methylation of the Mov-1 M-MuLV DNA have been observed using other enzymes (Ava I, Xho I) for target and non-target tissues (data not shown; Stuhlmann, H., Jaehner, D., and Jaehnisch, R., in preparation). Further analyses of tumor DNA by Msp I/Hpa II, Hha I, Sal I, Ava I, Sma I, and Xho I revealed that somatically acquired proviruses are hypomethylated at these sites when compared to the genetically transmitted Mov-1 M-MuLV DNA from both tumor and non-tumor tissues.

In this study we analyzed whether hypomethylation of somatically acquired MuLV genomes is correlated with moderate or hypersensitivity to DNase I. Also, the extent of methylation at Hpa II/Msp I sites was determined for the genetically transmitted Mov-1 M-MuLV DNA. Therefore, Eco RI DNA fractions containing several of the reintegrated proviral sequences were isolated and cleaved by Kpn I. This enzyme yields differently sized fragments for authentic and recombinant proviral genomes (Fig. 2)(5); Eco RI-Kpn I fragments of 3.6 and 1.8 kbp are diagnostic for recombinant MuLV DNA whereas authentic genomes yield fragments of 2.7 and 1.3 kbp respectively (Fig. 2). In addition each Kpn I digested Eco RI DNA fraction was cleaved by Hpa II and Msp I respectively (Fig. 6). Hpa II and Msp I are isoschizomeres from which Msp I cleaves the recognition sequence independant whether this site is methylated or not (31). Our analyses revealed that both authentic and recombinant reintegrated M-MuLV genomes are largely hypomethylated at the Hpa II/Msp I recognition sequences when compared to the Mov-1 M-MuLV genome (Fog. 6, Panel A) which is extensively methylated at these sites.

Although only some of the reintegrated proviral copies display flanking chromatin regions hypersensitive to DNase I, these proviral DNAs display grossly a similar extent of hypomethylation at their Msp I/Hpa II sites when compared to the proviral copies moderately sensitive to DNase I. Similar results were obtained after analyzing Eco RI DNA fractions directly with Hpa II/Msp I (data not shown). Since all somatically acquired MuLV sequences are moderately sensitive to DNase I (Fig. 1) the data might suggest a correlation of this moderate sensitivity to DNase I and hypomethylation. There is no direct correlation between hypersensitivity to DNase I and hypomethylation of reintegrated proviral genomes at the Msp I/Hpa II sites. The data do, however, indicate that

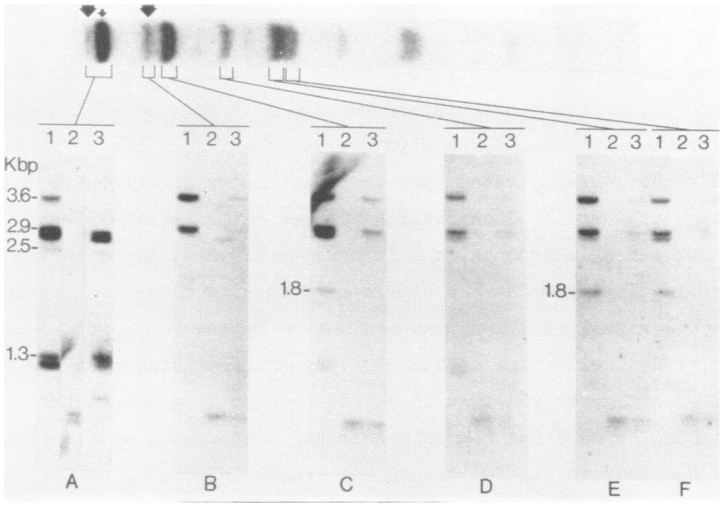

Fig. 6. Methylation at Hpa II/Msp I restriction sites in tumor tissue DNA. Several Eco RI DNA fractions, containing Mov-1 M-MuLV DNA or reintegrated proviral sequences were analyzed by Southern blotting after cleavage with Kpn I (lanes 1), Kpn I + Msp I (lanes 2), and Kpn I + Hpa II (lanes 3), by hybridization to a M-MuLV specific cDNA probe. Panel A shows the results for the Mov-1 M-MuLV containing fraction (indicated by a small arrow); this fraction also contains a 5'-end of a recombinant provirus, indicated by the characteristic 3.6 kbp Eco RI-Kpn I DNA fragment (Fig. 2). The faint hybridizing fragment of 2.5 kbp represents the 5'-virus-cellular DNA junction fragment of the Mov-1 M-MuLV DNA, whereas the fragments around 2.9 and 1.3 kbp are characteristic internal fragments of M-MuLV proviral DNA (Fig. 2)(5). Panels B-F show analyses of reintegrated proviral DNAs; the 1.8 kbp fragment (Panels C, E, and F, lanes 1) is derived from the 3'-end of recombinant proviral DNA (Fig. 2). Fragments are further indicated as in Fig. 2.

each tumor contains at least one recombinant proviral MuLV genome with the features characteristic for "active" chromatin: a) a DNase I hypersensitive configuration, and b) hypomethylation. Therefore, the results support the hypothesis that expression of MCF-encoded geneproduct(s) is essential for the maintenance of the transformed state.

DISCUSSION

Several DNase I probing studies of chromatin have provided convincing evidence that "active" chromatin has an altered, more accessible configuration (32). Such DNase I sensitive conformations have been observed for the globin gene sequences in nuclei of cells actively expressing these genes (20,21),for the ovalbumin gene in the hen oviduct (33), for rDNA in various organisms, and

for integrated viral genomes of both DNA and RNA tumor viruses (for review see 22), for heat-shock loci in Drosophila tissue culture cells (24,26), and for chromatin sequences complementary to a total population of nuclear RNA (22).

This more accessible configuration results in a more DNase I sensitive chromatin structure when compared with overall nontranscribed chromatin. More recent studies have demonstrated that there are sites in chromatin which are hypersensitive to DNase I. Such sites have been mapped at the 5'-ends of several heat-shock genes in Drosophila melanogaster (24,26). Also, embryonic chick-red-blood-cells display a DNase I hypersensitive site close to the beginning of the embryonic β-globin (21) and presumed α-type U-gene (23); both embryonic and definitive red cells show a DNase I hypersensitive site near the 5'-end of both α-type globin genes (23). Further, the Drosophila melanogaster histone gene repeat also exposes DNA segments near the 5'-terminus of all five histone genes (27). Recently it was found that the transcriptionally active endogenous retrovirus locus ev-3 in chick cells also contains DNase I hypersensitive sites in each of its two long terminal repeats while the inactive ev-1 locus does not display such sites (34). These results suggest that mainly sequences around promoter regions of actively transcribed genes are hypersensitive to DNase I whereas the coding sequences are not. Coding sequences of actively transcribed genes, however, are always found in chromatin conformations more sensitive to DNase I than non-transcribed genes.

In order to probe for potentially active proviruses we have determined the DNase I sensitivity of genetically transmitted and somatically acquired proviruses in M-MuLV-induced tumors. These studies revealed that the genetically transmitted Mov-1 M-MuLV genome in both tumor and non-tumor tissues of Balb/Mo mice is not in a DNase I sensitive conformation. The genetically transmitted endogenous AKR-MuLV genome (4,8) and other endogenous viral sequences that can cross-react with AKR-MuLV cDNA (8) are also not in DNase I sensitive conformations in these tissues from both Balb/Mo and Balb/c mice. The non-viral, cellular homologue of Mo-MSV specific mos-sequences (30) was also found in a DNase I resistant conformation in chromatin from M-MuLV-induced tumors. The somatically acquired MuLV sequences in M-MuLV-induced tumors of Balb/Mo and Balb/c mice can be devided in two classes according to their sensitivity to DNase I: most proviral DNAs display a moderately DNase I sensitive conformation whereas some expose a DNase I hypersensitive configuration at their 5'-end. A new fragment of 7.0 kbp arises after DNase I digestion of chromatin and subsequent cleavage with Eco RI. This fragment contains sequences derived from the 5'-part of recombinant proviruses: from the 5'-LTR up to the Eco RI site at 6.9 kbp. A DNase I

hypersensitive configuration is located probably slightly in front of the 5'-LTR. No 3'-ends of recombinant proviruses and their 3'-flanking cellular sequences have been found to be hypersensitive to DNase I. Also, the analyses did not reveal whether reintegrated authentic M-MuLV genomes display DNase I hypersensitive configurations since none of the tumors analyzed contained an Eco RI DNA fraction comprizing exclusively an authentic proviral genome. Other restriction enzymes did also not solve this issue. The results indicate that some reintegrations occur near DNase I hypersensitive chromatin regions or these reintegrations generate in some instances DNase I hypersensitive configurations in chromatin at their site of insertion. Although this issue remains unsolved for the moment the results clearly show that tumor tissues contain recombinant MuLV genomes located in "active" chromatin regions.

The function of the exposed sequences at the 5'-ends of proviral DNA is still obscure. Previously, exposed sequences have been mapped at the initial portion of the mRNA leader sequences of the histone genes H3, H4, H2A, and H2B in Drosophila melanogaster (25). Also, the putative regulatory sequence near the center of both the Drosophila and Xenopus 5S RNA genes is packaged in an exposed linker configuration (35). Therefore, the exposed sequences might represent interaction sites with specific regulatory proteins or present exposed DNA sequences for binding of RNA-polymerase. Weintraub (27) postulated that regions of DNase I hypersensitivity may be involved in orienting regulation signals on DNA with respect to their associated nucleosomes and also with respect to the coiling of the DNA in the chromosome fiber (27). A remarkable feature of the DNase I hypersensitive sites juxtaposing the recombinant proviruses is their location: 400-500 nucleotides before the TATA-box and Cap-site in the 5'-LTR (36). A subdomain of chromatin defined by a DNase I hypersensitive site approximately within 500 bp before the 5'-end of the first α-globin gene in the active α-gene cluster in definitive erythrocytes of chickens has been reported recently (23).

The relative broadness of the band, representing the newly emerging fragment at around 7.0 kbp after DNase I digestion and Eco RI cleavage (Fig. 1) suggests the presence of several closely positioned DNase I hypersensitive sites in front of or at the start of the 5'-LTR of some recombinant proviruses. Such closely positioned sites, which can span up to 300 bp, have also been found at the 5'-ends of heat-shock genes in Drosophila melanogaster (24,26).

Since hypomethylation has also been postulated as a characteristic, though not sufficient marker associated with active genes (23,37-42) we have also determined the extent of base methylation at Hpa II/Msp I recognition sequences.

All somatically acquired authentic and recombinant MuLV genomes are hypomethylated (as observed previously for somatically acquired M-MTV sequences in carcinomas; 43) whereas the genetically transmitted M-MuLV copy is extensively methylated at the Hpa II/Msp I recognition sequences. MuLV proviral copies that display a hypersensitive site to DNase I at their 5'-end display grossly the same extent of methylation at their Hpa II/Msp I recognition sequences as compared to copies moderately sensitive to DNase I. All reintegrated proviral DNAs are at least moderately sensitive to DNase I; this might therefore suggest a correlation between moderately DNase I sensitive sequences and hypomethylation. It has been reported for other genes that sequences sensitive to DNase I are hypomethylated, whereas low-level methylation does not a priori imply DNase I sensitivity (33,39). The lack of extensive methylation of somatically acquired sequences might simply be due to inefficient de novo methylation and not be related to chromatin conformation. We have also observed that during aging of a Balb/c mouse the extent of methylation at the Hpa II/Msp I sites of endogenous viral sequences (those that can cross-react with AKR-MuLV cDNA) is deminished while these sequences are not preferentially digested by DNase I.

Previously, it was shown that the Mov-1 M-MuLV DNA is hypomethylated at Sal I and Sma I sites in target tissues as compared to non-target tissues (5), although this proviral DNA is extensively methylated at the Hpa II/Msp I sites in tumor tissues. Also, the Mov-1 M-MuLV proviral DNA is not preferentially digested by DNase I in tumor tissues. These data suggest that this locus is inactive in transcription in tumor tissues.

In conclusion, tumors of different mice contain variable numbers of reintegrated recombinant and authentic M-MuLV genomes. A certain recombinant proviral genome (Fig. 2) seems to be required for transformation (5,8). Whether these proviruses exert their transforming activity by promoter-insertion or via a recombinant env-gene product is still obscure. However, those recombinant MuLV copies, displaying a flanking cellular DNA region hypersensitive to DNase I, are probably the best candidates for proviruses active in transcription. Therefore, these copies are most likely responsible for leukemic transformation. Cloning of these proviral sequences, selected by virtue of their DNase I hypersensitive configuration, will hopefully enable us to study the role of these recombinant genomes and flanking cellular sequences in the transformation process.

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