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Chromatin Conformation of Integrated Moloney Leukemia Virus DNA Sequences in Tissues of BALB/Mo Mice and in Virus-Infected Cell Lines

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The technique of preferential DNase I digestion of transcriptionally active chromatin regions was used to study the structural organization of integrated Moloney murine leukemia virus (M-MuLV) proviral sequences in various cells carrying integrated viral genomes. BALB/Mo mice, which carry M-MuLV as an endogenous virus at a single Mendelian locus, were used to examine the genetically transmitted viral genome copy and additional M-MuLV sequences acquired somatically during leukemogenesis. It has been shown previously that M-MuLV genome expression in these mice is restricted to lymphatic target tissues. In young homozygous BALB/Mo mice carrying one M-MuLV genome copy per haploid mouse genome in all cells we found that the genetically transmitted viral genome copy was in a preferentially DNase I-sensitive conformation in lymphatic target tissues, whereas in nontarget tissues the same sequence was not preferentially DNase I sensitive. This suggests that the chromatin conformation and the transcriptional activity of the integrated proviral genome are related to and probably determined by the state of cellular differentiation. In target tissues from BALB/Mo mice examined at different ages and in different stages of leukemogenesis the majority of the new somatically acquired M-MuLV sequences were preferentially DNase I digestible. A very similar pattern of DNase I digestibility was observed in target tissues from BALB/c mice exogenously infected with M-MuLV. This shows that in these tissues somatically acquired proviral sequences integrate preferentially or exclusively at sites of the host genome in which they are in a transcriptionally active chromatin conformation. Alternatively, the chromatin structure of the respective host genome region may be changed after the integration of viral DNA. In nontarget tissues from BALB/Mo mice the M-MuLV-specific sequences remained DNase I resistant throughout the lives of the animals. A different pattern of DNase I digestibility was observed in virus-infected cell lines which had been produced by low-multiplicity infection, cloned, and selected for virus production. When cell lines harboring different numbers of M-MuLV proviral copies were examined, it was found that a minority of the proviral sequences (on the average only one M-MuLV genome copy per haploid mouse genome) were preferentially digestible by DNase I, independent of the total number of proviral genome copies present. This suggests that the chromatin conformation of newly acquired proviral sequences is influenced by the state of differentiation of the infected cell or the way infected cells are selected or both.

The eucaryotic genome is organized in basic chromatin subunits (nucleosomes) which consist of DNA complexed with a stable complement of histones and a variety of nonhistone chromosomal proteins (for a recent review, see reference 15). The elucidation of the structure of the chromatin and the specific interactions between DNA and chromatin proteins is a prerequisite for understanding the control of gene expression. The concept that structural changes occurring within the chromatin complex are responsible for the activation of specific genomic regions and that these changes are reflected by differences in the sensitivity of these regions toward nucleolytic enzymes can be exploited experimentally. From various studies following this concept it has been discovered that transcription of a specific chromatin region is accompanied by a disruption of some of the organizational features of the chromatin, resulting in an increased DNase sensitivity of actively transcribed genes (6, 16-19, 27, 28, 34, 35).

Retroviruses can serve as model systems for studying the structural organization of the eucaryotic genome and the regulation of gene expression. As endogenous viruses they are part of the normal genetic complement of a wide variety of animal species. Alternatively, as exogenous viruses they are not part of the genetic information of their host but can, after infection of a susceptible cell, integrate into the genome of the host cell, where they serve as template for the transcription of virus-specific RNA (for recent reviews, see references 1 and 5). The expression of the integrated proviral genome is linked to the differentiated state of the infected cell (26) and is controlled by genetic factors of the host (31). One of the most intriguing properties of retroviruses is their ability to integrate their genetic information into the host cell genome. Although widely studied, the details of this process are only poorly understood. Of special interest are the factors determining the sites at which a proviral genome can integrate into the host cell DNA and the possible relationship between the integration site and the expression of the proviral genome.

Recently, a mouse line (BALB/Mo) which carries the Moloney murine leukemia virus (M-MuLV) as an endogenous virus at a single Mendelian locus has been established (22). This locus (*Mov-1*) has been genetically mapped on chromosome 6 of the BALB/Mo mouse genome (8). BALB/Mo mice express the M-MuLV genome in lymphatic target tissues (spleen, thymus) and develop a thymus-derived leukemia. Viral gene expression and leukemogenesis are accompanied by an increase in virus-specific DNA sequences and by the reintegration of additional M-MuLV proviral genome copies into the DNA of target tissues. In nontarget tissues (liver, brain, muscle) the viral genome is not expressed, and no increase in virus-specific DNA sequences can be observed (24). Thus, these mice offer the unique opportunity to study the structural organization of a well-defined proviral DNA sequence in various states of expression.

We have previously shown that the difference between transcriptional activity of the M-MuLV proviral genome in target and in nontarget cells from BALB/Mo mice is reflected by a different DNase I sensitivity of the viral sequences: in spleen cells M-MuLV sequences are preferentially digestible by DNase I, whereas in liver cells the M-MuLV-specific sequences are not preferentially sensitive toward DNase I (9). However, these experiments were performed with old leukemic mice containing in their target cells three to four M-MuLV genome copies power haploid mouse genome (22, 24). Only two of the copies were DNase I sensitive, whereas the remaining one to two copies showed the same relative resistance toward DNase I as did the overall host DNA sequences. These experiments did not discriminate between the multiple proviral copies in target cells and therefore did not necessarily compare the conformation of identical chromatin regions in expressed and nonexpressed conformations and revealed no information about the transcriptional activity of the genetically transmitted M-MuLV copy and the additional sequences acquired somatically during leukemogenesis. In this publication we describe additional DNase digestion experiments with BALB/Mo mice of various ages and in different stages of leukemogenesis. We also studied the chromatin conformation of M-MuLV proviral sequences in target tissues from BALB/c mice exogenously infected with this virus and in fibroblast cell lines infected with M-MuLV in vitro and harboring different numbers of proviral genome copies.

MATERIALS AND METHODS

Cells and virus. All cell lines were grown in Dulbecco-modified Eagle medium supplemented with 5 to 10% fetal calf serum (14). M-MuLV clone 1 was grown, purified, and tested as described previously (13).

Preparation of cell nuclei. All manipulations were carried out at 0 to 4°C. Mouse tissues were rinsed with ice-cold phosphate-buffered saline, minced, and homogenized in a motor-driven Teflon homogenizer in 0.5 M sucrose in buffer I (0.9 mM $CaCl_2$–0.9 mM $MgCl_2$–5 mM Tris-hydrochloride [pH 7.5]–25 mM KCl–0.14 mM spermidine). The sucrose concentration was then adjusted to 1.5 M, and the nuclei were isolated by centrifugation through a cushion of 2.2 M sucrose in buffer I. Centrifugation was either in the Beckman SW27 rotor at 25,000 rpm for 20 min or in the SW50.1 rotor at 20,000 rpm for 20 min. Nuclei were washed once in buffer I and resuspended in the same buffer at a DNA concentration of 1 mg/ml. Nuclei from tissue culture cells were isolated by swelling the cells in buffer II (10 mM Tris-hydrochloride [pH 7.5]–10 mM KCl–5 mM $MgCl_2$). After 10 min the cells were broken in a glass homogenizer, and Nonidet P-40 was added to a concentration of 0.75%. Nuclei were sedimented, washed several times in buffer I, and resuspended in buffer I at a DNA concentration of 1 mg/ml.

DNase digestions. DNase I (Boehringer) or micrococcal nuclease (Sigma) were used at concentrations of 10 Kunitz units per ml each. Incubation was at 37°C. The percentage of DNA remaining $HClO_4$ precipitable was determined as described previously (9).

DNA purification. Nucleic acids were extracted by the method of Kirby (25) and precipitated with ethanol. DNA was further purified by cesium chloride-ethidium bromide equilibrium centrifugation as described previously (22, 23). In most experiments the

CsCl centrifugation was omitted to prevent a preferential loss of small DNA fragments in the DNase-digested samples. Instead, the DNA was further purified by sequential digestion with RNase and proteinase K and by extraction with chloroform-isoamyl alcohol (25:1). The results (hybridization) obtained with both isolation procedures in parallel experiments were not significantly different. All DNA samples were boiled for 15 min in 0.2 M NaOH containing a high salt concentration (either ~4 M CsCl or 2 M NaCl). Sizing of the DNA preparations on agarose gels showed that by this procedure undigested and DNase-digested DNA samples were sheared to fragments with similar size distributions. After boiling, the DNA was neutralized and finally precipitated twice with ethanol.

Preparation of M-MuLV-specific cDNA and molecular hybridization. The in vitro synthesis of M-MuLV complementary DNA (cDNA) by the endogenous reverse transcription reaction, the selection of M-MuLV-specific cDNA by the removal of all sequences cross-hybridizing with endogenous mouse viruses, and the quantitation of M-MuLV-specific sequences in mouse DNA in a cDNA excess hybridization reaction were done essentially as described previously (4, 23; see Fig. 2).

RESULTS

We carried out DNase digestions of native chromatin in situ to compare the structural organization of M-MuLV proviral DNA sequences in various virus-infected cells. Cell nuclei were isolated and incubated in the presence of DNase as described above. The extent of DNase digestion was monitored by determining the perchloric acid-soluble and -precipitable fractions. A typical DNase digestion kinetics diagram is shown in Fig. 1. After 3 h of digestion reproducibly 50 to 60% of the total DNA was rendered $HClO_4$ soluble. The extent of DNase digestibility was found to be identical for all mouse tissues examined (liver, spleen, thymus, tumor) and for both enzymes used (DNase I, micrococcal nuclease). In the various fibroblast cell lines the total DNase digestibility was usually 40 to 50%. It has been shown previously by the addition of fresh enzyme after 1 h of incubation that the undigested DNA fractions are resistant to further DNase digestion (9, 34). Before and after various times of DNase digestion samples of the incubation mixture were withdrawn, and the total undigested DNA was extracted and purified. The relative concentrations of M-MuLV-specific sequences in the DNA preparations were determined by hybridization with an M-MuLV-specific cDNA in a quantitative cDNA excess hybridization assay (4, 9, 23). Control experiments revealed that under the conditions used the hybridization reaction reached saturation in time (Fig. 2a). Figure 2b shows cDNA excess titration curves with standard DNAs from heterozygous and homozygous BALB/Mo embryos and from virus-producing and non-virus-producing BALB/Mo fibroblasts, respectively. Hybridizations with these DNAs were performed in every hybridization experiment and used as standards for the calculation of the concentrations of M-MuLV-specific proviral sequences in the DNA preparations.

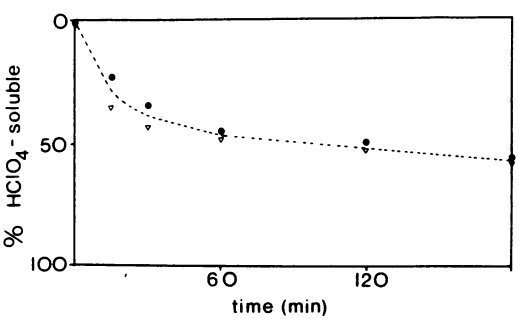

FIG. 1. *Kinetics of DNase digestion of chromatin in mouse cell nuclei. Nuclei were isolated from the spleen (●) and liver (▽) of a 2-month-old BALB/Mo mouse and digested with DNase I, and the $HClO_4$ solubility was determined as outlined in the text.*

Following this experimental protocol in all experiments, we examined the conformation of M-MuLV proviral sequences in a variety of virus-infected cells, including the following: (i) target and nontarget cells from BALB/Mo mice of different ages and in different stages of leukemogenesis; (ii) target cells from BALB/c mice exogenously infected with M-MuLV; and (iii) fibroblast cell lines infected with M-MuLV in vitro.

DNase I sensitivity of M-MuLV sequences in target and nontarget tissues from BALB/Mo mice. Young homozygous BALB/Mo mice carry one M-MuLV genome copy per haploid mouse genome in both their target (spleen, thymus) and nontarget (liver) tissues. This copy corresponds to the genetically transmitted viral genome which has been mapped on chromosome 6 (8). For comparing the conformation of these sequences in target and nontarget tissues, nuclei were isolated from spleen, thymus, and liver cells of 5- to 6-day-old homozygous BALB/Mo mice, and the DNase I digestibility of M-MuLV-specific sequences was determined. M-MuLV-specific sequences in spleen and thymus cells of such mice were preferentially sensitive toward DNase I, whereas the virus-specific sequences in liver cells from the same animals were as sensitive to DNase I as was the overall cellular DNA (Fig. 3a). Likewise, in target cells from young heterozygous BALB/Mo mice with 0.5 M-MuLV genome copies per

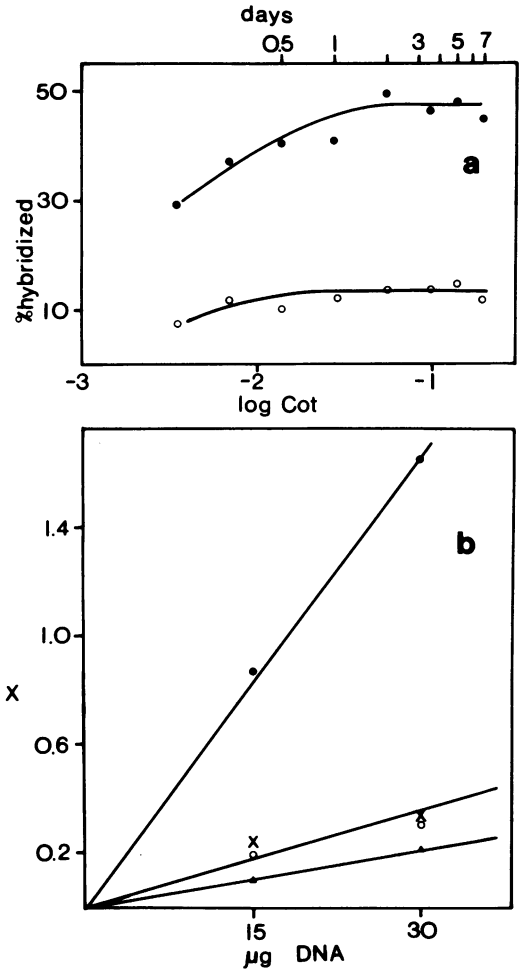

FIG. 2. *Determination of M-MuLV-specific DNA sequences by cDNA excess hybridization. (a) Hybridization kinetics. A total of 300 μg each of homozygous BALB/Mo embryo DNA (○) and leukemic BALB/Mo spleen DNA (●) were annealed with 7.5 × 10^{-4} μg of M-MuLV-specific radioactive cDNA at 68°C in 1 M NaCl. At the indicated times samples were withdrawn, and the level of hybridization was determined by S1 nuclease digestion. The abscissa indicates the C_0t calculated for the M-MuLV cDNA sequences. (b) Titration curves with standard DNAs. The indicated amounts of DNA from heterozygous (△) and homozygous (○) BALB/Mo embryos and from non-virus-producing (×) and virus-producing (●) BALB/Mo fibroblasts were annealed with 7.5 × 10^{-5} μg of M-MuLV-specific cDNA for 6 days under the conditions described above. The fraction X of M-MuLV-specific sequences in the cell DNA as compared with the input cDNA was computed from the equation % hybridized/100 = X/(1 + X) and used to determined the number of M-MuLV genome copies per haploid mouse genome equivalent as described previously (4, 23). The percentage of hybridization after correction for background hybridization to BALB/c DNA (4 to 6%) and*

haploid mouse genome equivalent the M-MuLV sequences were preferentially digestible by DNase I (Fig. 3b).

Additional experiments with young homozygous and heterozygous BALB/Mo mice are summarized in Tables 1 and 2. These experiments reproducibly showed that the genetically transmitted copy of the viral genome is in a DNase I-sensitive chromatin conformation in target cells of young BALB/Mo mice, whereas in nontarget cells the same sequences are DNase I insensitive. We conclude from these experiments that the chromatin conformation and thus probably the

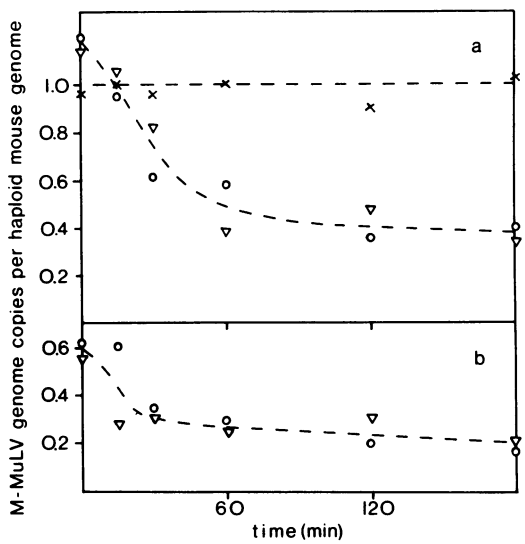

FIG. 3. *DNase I digestion of M-MuLV-specific sequences in chromatin from target and nontarget tissues of young BALB/Mo mice. Nuclei from livers (×), spleens (○), and thymuses (▽) of 5- to 6-day-old BALB/Mo mice were digested with DNase I. (a) Homozygous animals. (b) Heterozygous animals. Between 52 and 59% of the total DNA was $HClO_4$ soluble after 3 h of DNase digestion. After the indicated times samples of the incubation mixtures were withdrawn, and the total undigested DNA was extracted and purified as described in the text. The relative concentrations of M-MuLV-specific sequences were quantitated in the DNA preparations by a cDNA excess hybridization (see Fig. 2). The ordinate represents M-MuLV genome copies per weight of undigested DNA expressed as a haploid mouse genome equivalent.*

for maximum hybridization to M-MuLV 70S RNA (80 to 90%) in this experiment was between 9.3% (for 15 μg of heterozygous BALB/Mo embryo DNA) and 26% (for 30 μg of homozygous BALB/Mo embryo DNA). The DNA from virus-producing BALB/Mo fibroblasts hybridized to 46% (15 μg) and 62% (30 μg). In this and all subsequent experiments the cDNA input was 1,500 to 2,000 cpm, and the S1 resistance was between 150 and 700 cpm.

TABLE 1. *DNase I-sensitive and -resistant M-MuLV genome copies in target and nontarget cells from homozygous BALB/Mo mice*[a]

DNA source	Age of mice	Total no. of copies	M-MuLV genome copies that were		% Resistant
			DNase I sensitive	DNase I resistant	
Spleen	2 days	1.15	0.86	0.29	25
Spleen	6 days[b]	1.26	0.88	0.38	30
Spleen	10 days	1.42	0.8	0.6	42
Thymus	10 days	1.0	0.4	0.6	60
Thymus	15 days	1.09	0.74	0.35	32
Spleen	21 days[b]	1.56	0.95	0.61	39
Spleen	2 mo[b]	2.3	1.47	0.83	36
Spleen	3 mo[b]	2.5	1.67	0.83	33
Spleen	Leukemic[b]	3.0	1.5	1.5	50
Spleen	Leukemic[b]	3.3	1.92	1.38	42
Spleen	Leukemic[b]	3.7	2.0	1.7	46
Liver	2 days	1.15	0.2	0.95	83
Liver	21 days[b]	0.9	0	0.9	100
Liver	Leukemic[b]	1.0	0	1.0	100

[a] Cell nuclei were incubated with DNase I, and the M-MuLV genome copies in undigested and DNase I-digested DNA were determined as outlined in the legend to Fig. 3 and in the text. Of the total DNA, 50 to 60% was $HClO_4$ soluble after 3 h of digestion (compare with the text). Total M-MuLV genome copies represent the virus-specific sequences in undigested DNA; DNase I-resistant copies represent a mean of the results obtained after 2 and 3 h of DNase digestion.

[b] These experiments were performed with individual animals; in all other experiments pools of animals were used.

TABLE 2. *DNase I-sensitive and -resistant M-MuLV genome copies in target and nontarget cells from heterozygous BALB/Mo mice*[a]

DNA source	Age of mice	Total no. of copies	M-MuLV genome copies that were		% Resistant
			DNase I sensitive	DNase I resistant	
Spleen	5 days	0.62	0.48	0.14	23
Thymus	5 days	0.56	0.39	0.17	30
Spleen	7 days	0.61	0.43	0.18	30
Spleen	13 days	1.12	0.62	0.5	45
Spleen	29 days	1.18	0.87	0.31	26
Spleen	Leukemic[b]	1.7	1.1	0.6	35
Tumor	Leukemic[b]	2.0	1.26	0.74	37
Liver	Leukemic[b]	0.5	0	0.5	100

[a] For details see footnote *a*, Table 1.

[b] See footnote *b*, Table 1.

transcriptional activity of the integrated viral genome are determined by the state of cellular differentiation.

It has been shown previously that leukemogenesis in BALB/Mo mice is accompanied by an increase in M-MuLV-specific sequences in the target organs and that the new somatically acquired proviral genome copies are also integrated into the host cell genome (9, 22, 24; D. Jaehner, H. Stuhlmann, and R. Jaenisch, Virology, in press). To determine whether the newly acquired viral genome copies are in an active or inactive chromatin conformation, we carried out DNase I digestions of chromatin from different tissues of BALB/Mo mice in the preleukemic and leukemic phases of their lives. The results of many experiments with animals of different ages are summarized in Fig. 4 and Tables 1 and 2. In preleukemic BALB/Mo mice carrying approximately one (heterozygous animals) or two (homozygous animals) M-MuLV proviral genome copies in their target (spleen) cells, 60 to 70% of the viral sequences were DNase I sensitive. Similarly, in target and tumor tissues of leukemic mice with 1.7 to 2 (heterozygous animals) or 3 to 4 (homozygous animals) M-MuLV proviral genome copies integrated into their DNA, 50 to 70% of the virus-specific sequences were in a DNase I-digestible chromatin conformation (Fig. 4). In nontarget (liver) cells the M-MuLV-specific sequences remained DNase I resistant throughout the lives of the animals (Fig. 3 and 4; Tables 1 and 2). This preferential DNase digestibility of M-MuLV-specific proviral sequences in target cells was not observed when similar experiments were performed with micrococcal nuclease instead of with DNase I (9). These data confirm and extend our previous findings (9) and show that the majority of M-

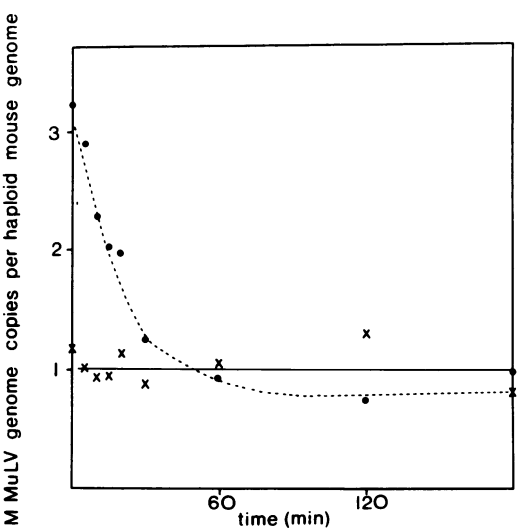

FIG. 4. *DNase I digestion of M-MuLV-specific sequences in chromatin from target and nontarget tissues of a leukemic, homozygous BALB/Mo mouse. Nuclei from a liver (×) and a leukemic spleen (●) of a 5-month-old BALB/Mo mouse were digested with DNase I. Of the total DNA, 56% (liver) and 57% (spleen) were $HClO_4$ soluble after 3 h of DNase digestion. For details see the legends to Fig. 2 and 3.*

MuLV-specific proviral genome copies integrated into the genome of target cells of preleukemic and leukemic BALB/Mo mice are in a DNase I-sensitive, i.e., transcriptionally active, chromatin conformation.

DNase I sensitivity of M-MuLV sequences in target cells from exogenously infected BALB/c mice. To determine whether exogenous infection with M-MuLV also leads to a preferential integration of the viral genome in a DNase I-sensitive conformation in target cells, we carried out DNase I digestions of chromatin from different tissues of BALB/c mice infected after birth with M-MuLV. We observed that the pattern of DNase I digestibility of M-MuLV sequences in the chromatin of spleen and tumor cells was very similar to that found in BALB/Mo mice: in young animals carrying about one M-MuLV genome copy per haploid mouse genome as well as in preleukemic and leukemic animals with two to five proviral copies present, 60 to 80% of the integrated viral sequences were in a DNase I-sensitive chromatin conformation. No viral sequences were detected in nontarget (liver) cells from M-MuLV-infected BALB/c mice (Table 3). This shows that the majority of somatically acquired M-MuLV-specific sequences in virus-infected BALB/c mice as well as in BALB/Mo mice are in a transcriptionally active chromatin conformation.

DNase I sensitivity of M-MuLV proviral sequences in infected fibroblast cell lines. To study the conformation of M-MuLV proviral sequences in nontarget fibroblasts, we carried out DNase I digestion experiments with a series of M-MuLV-infected cell lines, including virus-producing and non-virus-producing fibroblast cell lines established from homozygous BALB/Mo embryos (8) and virus-producing 3T3 cell lines harboring different numbers of M-MuLV proviral DNA copies. The latter lines were produced by low-multiplicity infection, cloned, and selected for virus production (14). Representative results of these experiments are shown in Fig. 5 and 6 and summarized in Table 4.

In non-virus-producing fibroblasts from BALB/Mo mice carrying one M-MuLV genome copy per haploid mouse genome (8) all the virus-specific sequences were found to be in a DNase I-resistant configuration. This is in agreement with the finding that these cells neither produce infectious virus nor transcribe virus-specific RNA. When these cells were superinfected with M-MuLV and subsequently produced high titers of virus, they acquired three to four new viral genome copies per haploid mouse genome (8), and some of the newly acquired M-MuLV proviral sequences were in a DNase I-sensitive configuration (Fig. 5; Table 4). An analysis of cell lines established by low-multiplicity infection in vitro and carrying different numbers of M-MuLV proviral genome copies revealed that in these cells a minority of the viral sequences (on the average only one M-MuLV proviral genome copy per haploid mouse genome) were in a DNase I-sensitive configuration. This was independent of the total number of proviral genome copies integrated into the cell line analyzed (Fig. 6; Table 4). An interesting result was obtained with cell line B7, which was examined twice: when this cell line was established, it contained 1.6 M-MuLV genome copies per haploid mouse genome equivalent. In one experiment, when frozen cells were thawed, they still contained 1.6 genome copies per haploid mouse genome, and

TABLE 3. *DNase I-sensitive and -resistant M-MuLV genome copies in target and nontarget cells from neonatally infected BALB/c mice*[a]

DNA source	Age of mice	Total no. of copies	M-MuLV genome copies that were		% Resistant
			DNase I sensitive	DNase I resistant	
Spleen	10 days	0.87	0.47	0.4	46
Spleen	14 days[b]	1.6	1.29	0.31	19
Spleen	22 days[b]	1.95	1.11	0.84	43
Spleen	Leukemic[b]	3.0	2.33	0.67	22
Spleen	Leukemic[b]	2.6	2.04	0.56	22
Tumor	Leukemic[b]	2.5	1.68	0.82	33
Tumor	Leukemic[b]	5.5	4.81	0.69	13
Liver[b]		0	0	0	0

[a] For details see footnote *a*, Table 1.
[b] See footnote *b*, Table 1.

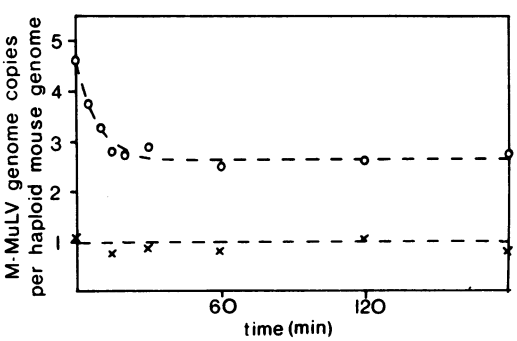

FIG. 5. *DNase I digestion of M-MuLV-specific sequences in chromatin from virus-producing and non-virus-producing BALB/Mo fibroblasts. Virus-producing* (○) *and non-virus-producing* (×) *BALB/Mo fibroblasts were prepared from homozygous BALB/Mo embryos, transformed by simian virus 40, and cloned to obtain permanent cell lines as described previously (8). Nuclei were isolated and purified and digested with DNase I. Of the total DNA, 43%* (○) *and 46%* (×) *were $HClO_4$ soluble after 3 h of DNase digestion. For details see the legends to Fig. 2 and 3.*

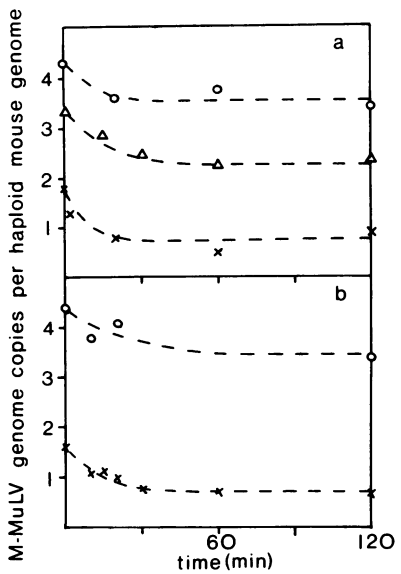

FIG. 6. *DNase I digestion of M-MuLV-specific sequences in M-MuLV-infected fibroblast cell lines. Fibroblast cell lines were infected with low multiplicities of M-MuLV and cloned, and virus-producing cell lines were selected (14). Nuclei were isolated and digested with DNase I. Between 40 and 50% of the total DNA was $HClO_4$ soluble after 2 h of DNase digestion. For details see the legend to Fig. 3. (a) Cell lines A9 (○), 1a (△), and C6 (×). (b) Cell line B7, experiments 1 (×) and 2 (○) (see the text).*

about 1 genome copy was sensitive to DNase I. However, when the experiment was repeated at a later time, the number of M-MuLV-specific sequences had increased to 4.4 genome copies, suggesting that superinfection had taken place in this case (14). However, the number of DNase I-sensitive M-MuLV sequences had remained constant, suggesting that the newly acquired viral sequences were in a DNase I-resistant configuration (Fig. 6; Table 4).

TABLE 4. *DNase I-sensitive and -resistant M-MuLV genome copies in M-MuLV-infected fibroblasts*[a]

Clone	Total no. of copies	M-MuLV genome copies that were		% Resistant
		DNase I sensitive	DNase I resistant	
C6	1.8	1.0	0.8	44
B7 I	1.6	0.9	0.7	44
B7 II	4.4	1.0	3.4	77
1A	3.6	1.0	2.6	72
A9	4.3	1.0	3.3	77
BALB/Mo$^-$	1.0	0	1.0	100
BALB/Mo$^+$	3.4	0.4	3.0	88
BALB/Mo$^+$	4.6	1.8	2.8	61

[a] B7 I and B7 II correspond to two experiments with this cell line (see text). BALB/Mo$^-$ and BALB/Mo$^+$ represent non-virus-producing and virus-producing BALB/Mo fibroblasts, respectively (8). Of the total DNA, 40 to 50% was $HClO_4$ soluble after 2 to 3 h of digestion (see text). For further details see footnote *a*, Table 1 and the legends to Fig. 5 and 6.

DISCUSSION

In the experiments described here we attempted to compare the structural organization of multiple M-MuLV proviral genome copies integrated into the DNA of a variety of virus-infected cells in vivo and in vitro with the technique of DNase digestion of native chromatin in isolated cell nuclei. The nuclease sensitivity of the viral sequences was monitored by molecular hybridization, using as a probe a selected cDNA which specifically detects M-MuLV sequences and does not hybridize to endogenous murine viruses (4, 23). Our results, therefore, directly reflect the fate of the M-MuLV genome and are not undermined by the presence of endogenous viral sequences, a difficulty frequently encountered by others doing similar experiments (19, 20, 28). Furthermore, it has been shown previously (9; Jaehner et al., in press) that the vast majority of M-MuLV-specific DNA sequences in lymphoid target tissues of BALB/Mo mice are present as integrated proviral DNA and that only very few unintegrated circles(<0.1 of a genome copy per cell in the tissues analyzed in the experiments described here) can be detected, facts which might complicate the interpretation of the data. The complexity of the cDNA used has so far not accurately been determined, but we assume that it represents approximately 30% of the viral genome (23, 32). Although, therefore, in a strict sense our experiments allow conclusions only about the state of this 30% of the viral genome, we take the results as being representative for the entire viral genome. Moreover, we assume that the preferential loss of M-MuLV-specific sequences during DNase I digestion is due to an altered chromatin structure of these sequences correlated to a transcriptional activity of the respective genomic region, in analogy to the findings by others (16–19, 28, 34). This assumption is further supported by the finding that no preferential degradation of M-MuLV-specific sequences can be observed either in nontarget (liver) cells or non-virus-producing fibroblasts (Fig. 3 to 5; Tables 1 to 3) or in micrococcal nuclease-digested chromatin from target and nontarget cells (9), although in all cases the DNA is degraded to the same extent and shows after our purification procedure a very similar size distribution.

DNase I digestion of chromatin from target

and nontarget tissues of young BALB/Mo mice revealed that the genetically transmitted copy of the M-MuLV genome (*Mov-1* [8]) was DNase I sensitive in target (spleen, thymus) cells, whereas the same sequence was DNase I resistant in nontarget (liver) cells (Fig. 3; Tables 1 and 2). This result confirms the idea that identical regions of the eucaryotic genome may have a different configuration in different tissues, depending on the state of expression of these regions (17, 34), and suggests that tissue-specific activation of M-MuLV expression in BALB/Mo mice (24) is correlated to a different chromatin structure of the integrated viral genome.

A series of experiments described here deals with the chromatin conformation of M-MuLV proviral genome copies amplified and reintegrated into the mouse genome during the preleukemic and acute phases of leukemogenesis. These experiments were undertaken to find out whether the new somatically acquired proviral genome copies are in an active or inactive chromatin conformation. Restriction enzyme analyses have shown that multiple integration sites exist on the host cell DNA for the M-MuLV provirus (3, 30, 32; Jaehner et al., in press) as well as for other retroviruses (2, 10, 12, 21). We show here that most (60 to 80%) of the M-MuLV proviral sequences in target tissues from preleukemic and leukemic BALB/Mo mice and from neonatally infected BALB/c mice are in a DNase I-sensitive chromatin structure (Fig. 4; Tables 1 to 3). This suggests that integration of somatically acquired M-MuLV proviral sequences into lymphoid tissues occurs preferentially or exclusively at sites with a transcriptionally active chromatin conformation. Alternatively, integration might occur randomly, and lymphoid target cells might respond to the integration of M-MuLV proviral sequences by an activation of the corresponding chromatin region.

A striking difference between the results obtained with leukemic, multiple M-MuLV proviral copies containing cells from BALB/Mo mice or from neonatally infected BALB/c mice was observed when we determined the DNase I digestibility of M-MuLV proviral sequences in infected fibroblast cell lines harboring different numbers of M-MuLV proviral copies: in these cell lines a minority (one genome copy per haploid mouse genome) of the M-MuLV genome copies were DNase I sensitive, irrespective of how many M-MuLV proviral genome copies the cells contained (Table 4; Fig. 5 and 6). This suggests that in these fibroblasts infected in vitro only one or a few of the integrated M-MuLV proviral genomes are transcriptionally active. In agreement with this interpretation is the fact that in these cell lines no correlation between the number of M-MuLV proviral copies and the amount of virus produced can be observed (14).

The experimental approach used here does not reveal which of the multiple viral genome copies in lymphoid target cells and virus-producing fibroblasts are DNase I sensitive. A solution to this problem has to await a further analysis by restriction enzyme digestion of partially DNase I-digested chromatin from these cells.

The difference observed between the conformation of proviral sequences in target cells in vivo and in nontarget cells in vitro is illustrated in Fig. 7, where we plotted the DNase I-resistant M-MuLV genome copies versus the total copies present in the cells examined. At present we have no explanation for this observation. It is tempting to speculate, however, that these results reflect a differentiation-specific difference in the mechanism of proviral integration: whereas in lymphatic cells the integration of the lymphotropic M-MuLV preferentially occurs at sites with an active chromatin conformation or results in an activation of this chromatin region, in fibroblasts integration might occur much more frequently at inactive sites or fail to result in an activation. As the fibroblast lines examined here were selected for virus production, one might predict that by low-multiplicity infection

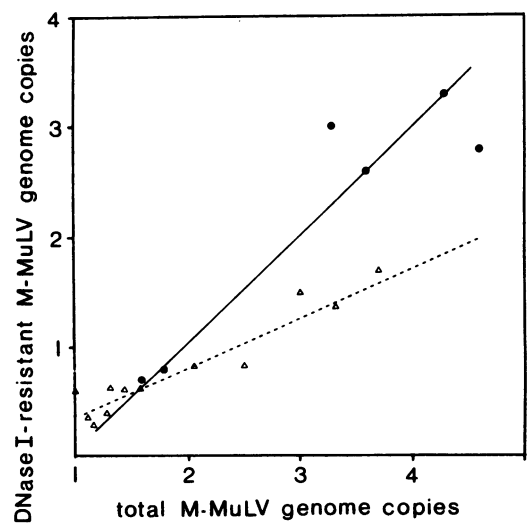

FIG. 7. *The fraction of DNase I-resistant M-MuLV genome copies in relation to the total genome copies in target tissues from BALB/Mo mice and in M-MuLV-infected fibroblasts. This figure summarizes the data for target tissues from homozygous BALB/Mo mice (△; Table 1) and for M-MuLV-infected fibroblasts (●; Table 4).*

of fibroblasts in vitro with high frequency cell clones can be obtained which contain but do not express proviral sequences, reminiscent of cells abortively transformed by simian virus 40 (29) or of "silent" infections of NRK cells with Rous sarcoma virus (7).

The interpretation of some of the results presented in this paper was complicated by the fact that even after extensive (3-h) DNase I digestion a certain fraction of M-MuLV-specific sequences always remained undigestible, i.e., large enough to form stable hybrids in our hybridization assay (Tables 1 to 3). There are several possibilities to explain this. (i) There may be heterogeneity in the cell populations examined. Our experiments with mouse tissues were usually performed with nuclei from total homogenized organs which might contain a mixture of virus-expressing and non-virus-expressing cells. (ii) Only part of the proviral genome(s) is expressed (i.e., is DNase I sensitive). If this were the case, the DNase I-resistant sequences should represent only a fraction of the sequences recognized by our cDNA probe. We tested this by hybridizing undigested and DNase I-digested BALB/Mo leukemic spleen cell DNA to our cDNA in cell DNA excess and found that both DNAs protected our cDNA to the same extent (83%), thus excluding this possibility. (iii) An actively transcribed region of eucaryotic chromatin may be in general preferentially but nevertheless only partially digestible by DNase I. This seems to us the most likely explanation. The reasons for the increased DNase I sensitivity of transcribed chromatin regions are only poorly understood. Recent work by Wu et al. (36) suggests that it is due to a disruption of organizational features of the chromatin on the supranucleosomal and, to some degree, on the nucleosomal level. However, it also seems likely that actively transcribed chromatin regions retain some degree of structural organization, leaving some DNA sequences inaccessible to nucleolytic enzymes. Another possibility is that chromosomal proteins liberated by the DNase digestion rebind to the undigested chromatin, thereby rendering it increasingly DNase resistant (11, 33). Most studies comparable to the ones described in this paper with other viral or nonviral genes have been performed under conditions of limited DNase digestion and with the aim of demonstrating preferential (and not total) digestibility of active genes by DNase I (16–19, 28, 34). It remains to be elucidated whether partial resistance of active chromatin regions toward this enzyme is a general phenomenon or is peculiar to our system. A further examination of this and the other questions discussed here may increase our understanding of the structural organization of eucaryotic chromatin in general and may be exploited in attempts to isolate specific transcriptionally active chromatin regions and characterize factors or events that lead to an activation of specific regions of the eucaryotic genome.

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