Analysis of Tissue-Specific Methylation Patterns of Mouse Mammary Tumor Virus DNA by Two-Dimensional Southern Blotting

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We used a two-dimensional Southern blotting procedure to analyze the tissue-specific methylation patterns of the five endogenous mouse mammary tumor viruses in the GR/A mouse strain. Our findings suggest that in certain tissues (brain, kidney, and liver) all proviruses are extensively methylated. In other tissues (spleen, placenta, and testes) all proviruses are hypomethylated to some degree. In these tissues individual proviruses display both quantitative and qualitative differences in methylation. We interpret the general patterns of tissue-specific hypomethylation in terms of a "hitch-hiker" model: mouse mammary tumor virus proviral methylation patterns reflect the tissue-specific activity of neighboring sequences. The observation that certain sites on particular proviruses are differentially methylated in a tissue-specific fashion may reflect tissue-specific differences in the makeup or conformation, or both, of proviral-containing chromatin.

Tissue-specific DNA methylation patterns have been observed for numerous cellular genes and for viruses in a variety of eucaryotes (4, 6, 16). In many instances the absence of methyl groups in DNA is correlated with gene expression. However, a substantial number of cases are now known in which hypomethylated genes are not actively transcribed (1). Two of the observations that have emerged from DNA methylation studies are especially pertinent in the present context: large regions of nontranscribed DNA flanking active genes may exhibit hypomethylation (20, 24), and the hypomethylation of specific sequences (e.g., promoters) may be critical for gene expression (13).

Recently we examined the methylation patterns of mouse mammary tumor virus (MuMTV) DNA in nonmammary tissues of several inbred mouse strains. Our results indicated that some nonmammary tissues (e.g., spleen, placenta, and testes) contained hypomethylated MuMTV proviruses and that other nonmammary tissues (e.g., brain, kidney, and liver) contained MuMTV proviruses in a highly methylated state (11). The rationale behind these experiments was to identify tissues in which MuMTV proviruses might be capable of transcriptional activity. Such information is of potential interest in light of recently published findings that indicate transcription and amplification of MuMTV proviruses in certain nonmammary neoplasias (5, 15). Since nonmammary tissues with hypomethylated MuMTV DNA did not contain detectable MuMTV RNA, we proposed that proviral DNA was hypomethylated due to its proximity to actively transcribed cellular sequences ("hitch-hiker" effect [11]).

The GR/A mouse strain harbors five endogenous, genetically transmitted MuMTV proviruses. Thus, if the methylation patterns of these proviruses are indeed determined by the transcriptional state of neighboring genes, we might expect that each provirus would exhibit a tissue-specific

methylation pattern. Because MuMTV proviral sequences are highly conserved in inbred mice, our initial attempts to distinguish one provirus from another by standard restriction enzyme digestion-Southern blotting procedures proved futile (11). We therefore modified the two-dimensional gel procedure of Yee and Inouye (25) into a two-dimensional Southern blotting procedure capable of detecting single-copy sequences in a mammalian genome.

In this procedure genomic DNA is cleaved with an enzyme which generates unique fragments from many of the DNA sequences of interest. These fragments are separated on gels and digested in situ with a methylation-sensitive enzyme, and the resultant DNA fragments are separated on a second-dimension gel. A Southern blot of this gel provides a visualization (multigene fingerprint) of the methylation patterns of the individual members of a multigene family. We used this technique with the MuMTV proviruses in the GR/A mouse strain to establish that in tissue displaying MuMTV proviral hypomethylation (i) different proviruses are hypomethylated to differing extents, and (ii) individual proviruses display methylation patterns which are tissue specific. We believe that these results support a model in which MuMTV proviral hypomethylation in nonmammary tissues reflects the activity of neighboring genes.

MATERIALS AND METHODS

Tissue DNAs. Genomic DNA was extracted from GR/A mouse tissues by the proteinase K-sodium dodecyl sulfate-phenol-chloroform method as previously described (7). Labeled probes were a mixture of cloned MuMTV *Pst*I fragments (14) labeled by nick translation (17).

Blot analysis. One-dimensional Southern blots were performed as previously described (7). Two-dimensional Southern blots were done as follows. *Sac*I-cleaved genomic DNA was electrophoresed in a thin, horizontal 0.8% agarose gel. A strip (0.5 by 7 cm) which contained the separated DNA fragments was cut from the gel and immersed in a large volume of restriction buffer. After 5 to 7 h the gel strip was placed in a test tube containing 4 ml of restriction buffer plus

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FIG. 1. *SacI* profiles of MuMTV proviruses present in GR/A mice. (A) GR/A mammary tumor DNA (lane 2) and liver DNA (lane 3) were digested with *SacI*, Southern blotted, and probed with labeled MuMTV DNA. The numbers on the right designate the various MuMTV-containing *SacI* fragments. The correspondence between *SacI* bands and MuMTV proviruses is given in the text. Lane 1 is a ^{32}P -*lambda*-*HindIII* digest, and the sizes of the fragments (in kilobases) are given on the left. (B) A lane similar to that of lane 3 in (A) was cut from a thin one-dimensional gel and run, without further treatment, at right angles into a second gel whose composition was identical to the first gel. The gel was then Southern blotted and probed with labeled MuMTV DNA. The numbers above the bands correspond to those in part (A). A ^{32}P -*lambda*-*HindIII* digest was run in a marker lane of the second gel left in [B], and the sizes of several reference fragments are indicated.

50 U of *HhaI*. The tube was placed at 10°C for 24 h, after which another 50 U of *HhaI* was added. After an additional 16 h at 10°C the tube was incubated at 37°C for 8 to 10 h. The gel strip then was removed from the tube and embedded in a thick, horizontal 0.8% agarose gel. The gel buffer for the second dimension was identical to that used in the first dimension. The second-dimension run was done at 35 V for 14 to 18 h, after which the gel was treated and blotted as previously described (7). A complete description of the two-dimensional Southern blotting technique will be published elsewhere (T. G. Fanning and R. D. Cardiff, manuscript in preparation).

RESULTS

Two-dimensional blot analysis. Our initial attempts to determine how many and which MuMTV proviruses were hypomethylated in various GR/A mouse tissues made use of double digests with a restriction enzyme insensitive to DNA methylation plus a methylation-sensitive enzyme. We cleaved tissue DNAs with *SacI* alone (methylation insensitive) or with *SacI* plus *HhaI* (methylation sensitive) and then compared the MuMTV restriction patterns. However, because large numbers of MuMTV fragments were often seen after digestion with the *HhaI* enzyme and because *SacI* and *SacI*-*HhaI* fragments often comigrated, we were unable to determine which *HhaI* fragments were derived from specific *SacI* fragments. The two-dimensional technique overcame these problems.

SacI cleavage of GR/A mouse DNA results in a MuMTV restriction pattern containing eight prominent bands (Fig. 1A). Because of limitations in the current technology we only analyzed fragments of 4 kilobases or larger. These are designated as bands 1 to 6 (Fig. 1A). When a one-dimensional gel slice (e.g., Fig. 1A, lane 3) is run in a second dimension, the bands present in the first dimension form spots which lie on a diagonal (Fig. 1B). This occurs because

the two gel dimensions are identical in composition and because there are no changes in the molecular weights of the restriction fragments. If the first dimension is treated with a restriction enzyme and if one or more of the MuMTV-containing fragments are cleaved, the cleavage products will migrate below the diagonal because of their smaller size (Fig. 2 and 3).

We allocated the five endogenous MuMTV proviruses in the GR/A mouse strain to bands 1 to 6 (Fig. 1A) on the basis of several criteria. These include a comparison of the MuMTV restriction profiles of various mouse strains and the segregation of MuMTV proviral bands in backcrosses involving MuMTV-negative feral mice (MTV⁻ [3]) and a hybrid MTV⁻ × GR/A strain developed in this laboratory (D. W. Morris and R. D. Cardiff, manuscript in preparation). Thus, *SacI* band 1 contains sequences from both MTV-8 and MTV-19, band 2 contains sequences from MTV-2, band 3 contains sequences from both MTV-8 and MTV-20, band 4 contains sequences from MTV-19, band 5 contains sequences from MTV-3, and band 6 contains sequences from MTV-20.

Methylation patterns of individual MuMTV proviruses in GR/A nonmammary tissues. Two-dimensional Southern blotting patterns of MuMTV proviruses in various GR/A tissues are shown in Fig. 2 and 3. In each case we used the methylation-insensitive enzyme *SacI* in the first dimension and the methylation-sensitive enzyme *HhaI* in the second dimension. It previously was shown that MuMTV proviruses are largely methylated in GR/A kidney, brain, and liver (7, 8). This result is substantiated by the results shown here (Fig. 2A; brain and liver give essentially identical patterns). The only MuMTV-containing *SacI* band to show an altered mobility is band 6 (MTV-20). This result also has been confirmed by one-dimensional mapping (not shown). MTV-20 also is the only proviral locus exhibiting hypomethylation in GR/A lung tissue (10).

FIG. 2. Two-dimensional MuMTV proviral methylation patterns in normal nonmammary GR/A mouse tissues. Tissue DNAs were extracted from (A) kidney, (B) spleen, (C) placenta, and (D) testes. One-dimensional gels of *SacI*-treated tissue DNAs were run and before second-dimension electrophoresis were treated with *HhaI* as described in the text. The numbering system (bands 1 to 6) corresponds to that presented in Fig. 1A. ^{32}P -*lambda*-*HindIII* markers are at the far left.

GR/A spleen (Fig. 2B), placenta (Fig. 2C), and testes (Fig. 2D) all exhibit extensive hypomethylation of several MuMTV proviruses. These two-dimensional patterns demonstrate not only which proviruses are hypomethylated but also the degree of hypomethylation. Thus, in spleen the *SacI* fragment containing MTV-19 (Fig. 2B, band 4) has at least several hypomethylated *HhaI* sites, whereas those *SacI* fragments containing MTV-2, MTV-3, and MTV-20 (Fig. 2B, bands 2, 5, and 6, respectively) each have at least one hypomethylated *HhaI* site. In the GR/A placenta the *SacI* fragments containing MTV-2, MTV-3, MTV-19, and MTV-20

all appear to have at least several hypomethylated *HhaI* sites (Fig. 2C). A similar result is seen in GR/A testes (Fig. 2D), although in this tissue the *SacI* fragments containing MTV-3 and MTV-19 may be more methylated than those in placenta (cf. band 5 [MTV-3] and bands 1 and 4 [MTV-19] in Fig. 2C and D).

Our results suggest then that MTV-20 (or its flanking DNA) is hypomethylated to various degrees in many GR/A mouse tissues (Fig. 2). MTV-2, MTV-3, and MTV-19 (or their flanking DNAs) are either fully methylated in some tissues (Fig. 2A) or hypomethylated to various extents, and

FIG. 3. Two-dimensional MuMTV proviral methylation patterns in normal mammary gland (A) and in a mammary tumor (B) from the GR/A mouse. The procedures used to obtain these patterns are identical to those described in the legend to Fig. 2.

at different positions, in a variety of other tissues (Fig. 2B to D). We were unable to adequately analyze MTV-8 because both *SacI* fragments containing this provirus comigrate with other MuMTV-containing *SacI* fragments. However, it has been reported that MTV-8 is fully methylated in all adult mouse tissues (10). MTV-8-containing *SacI* fragments correspond to bands 1 and 3. Since some uncleaved material remains on the diagonal at these positions in each tissue we examined (Fig. 2), our data tend to support the claim (10) that MTV-8 is methylated in all adult mouse tissues.

Methylation patterns of individual MuMTV proviruses in GR/A mammary tissue. In addition to normal nonmammary gland tissues we also examined mammary gland and mammary tumor tissues by the two-dimensional blotting technique (Fig. 3). The methylation pattern in GR/A mammary gland (Fig 3A) is very similar to that in GR/A spleen (cf. Fig. 2B and Fig. 3A). One apparent difference is the additional fragments migrating in the 1.5- to 3-kilobase range below the MTV-2 *SacI* fragment (Fig. 3A, band 2). These fragments probably originate from newly acquired MTV-2 proviruses, which are amplified to a minor extent in GR/A mammary gland tissue (8).

There is a near total absence of MuMTV-containing *SacI* fragments remaining on the diagonal in a GR/A mammary tumor (Fig. 3B). This suggests that in this particular tumor all endogenous proviruses, as well as all newly acquired MTV-2 proviruses, are hypomethylated to a certain extent (the dark spots below band 2 [Fig. 3B] originate from the acquired, unmethylated MTV-2 proviruses). This is not true of every mammary tumor, since we mapped others which show some material remaining on the diagonal (not shown).

DISCUSSION

Attempts to demonstrate MuMTV transcription in a variety of normal nonmammary mouse tissues have proven equivocal (10, 11, 22). In some instances small amounts of MuMTV-related RNA sequences have been reported, but since the mouse genome contains copious amounts of MuMTV-like sequences (2) the exact interpretation of these results is unclear. In any event, MuMTV transcriptional activity appears to be extremely minimal, or nonexistent, in all normal nonmammary tissues which have been examined.

Given the fact that MuMTV proviruses are transcriptionally inactive in most mouse cells, then why should these proviruses display tissue-specific methylation patterns? Many earlier studies suggested a direct correlation between gene hypomethylation and gene expression (18). In recent years this simple idea has been replaced with a more complex, and certainly more realistic, set of correlations and hypotheses involving generalized hypomethylation, hypomethylation at specific sites, chromosomal position, tissue tropism, and gene expression (1, 4, 12).

MuMTV transcription is under hormonal control (19). This imparts a certain tissue tropism to MuMTV expression, and it seems reasonable to suppose that cellular factors involved in the hormonal control of MuMTV transcription are absent in many of the tissues in which we find hypomethylated proviruses. In addition, although many proviral CpG dinucleotides are unmethylated in these tissues, many others remain methylated (11). These methylated sites may lie in regions of the proviruses critical for transcription initiation, thus preventing gene expression (4, 12, 13).

Transcriptionally silent MuMTV proviruses exhibit remarkable tissue-specific methylation patterns (Fig. 2 and 3). We believe that these patterns can be attributed to another of the parameters known to play a role in DNA methylation,

chromosomal position. The role of chromosomal position in determining the methylation-DNase sensitivity patterns of cellular genes and of proviruses is well known (9, 12). Regions around some active cellular genes, extending many kilobases in both directions, exhibit DNA hypomethylation and increased sensitivity to DNases (12, 20, 24). In a previous report we speculated that MuMTV proviral methylation patterns reflected a hitch-hiker effect in which the proviruses adopted the methylation patterns and DNA conformations of those regions of the genome in which they happened to integrate. Tissue-specific expression of genes within these regions then would explain the MuMTV methylation patterns we observe (11). In an elegant study involving several mouse strains, as well as hybrids between them, Günzburg and Groner came to similar conclusions (10).

In addition to allowing us to identify which MuMTV proviruses are hypomethylated in GR/A mouse tissues, the two-dimensional blotting method allowed us to define the degree of hypomethylation at particular genomic sites. For example, the MTV-2 provirus shows strikingly different patterns in all the nonmammary tissues that we tested (compare the patterns under band 2 in Fig. 2A to D). The fact that MTV-2 exhibits a unique pattern in each mouse tissue demonstrates that specific sites on this provirus are less methylated in some tissues than in others. We do not know the reason for this type of site specificity, but possibilities include the following: (i) certain proviral sequences display tissue-specific conformational differences, (ii) certain proviral sequences display tissue-specific associations with a chromosomal protein(s), or (iii) both (i) and (ii). For example, DNA conformations other than B DNA (21), the organization of chromatin into complex superstructures (23), or both may influence cytosine methylation by making DNA more or less accessible to methylating-demethylating enzymes. An examination of Fig. 2 suggests that what is true for MTV-2 also applies to the other four MuMTV proviruses in the GR/A mouse.

We undertook the study of MuMTV proviral methylation patterns in nonmammary tissues to identify proviruses which might be transcriptionally active in certain normal mouse tissue, and a potentially interesting correlation has emerged from our studies. In the GR/A mouse strain, MTV-2 proviral transcription and amplification have been observed in T-cell lymphomas (15), and we show here, as well as in a previous publication (11), that virtually all tissues associated with the mouse lymphatic system display hypomethylated MTV-2 proviral sequences. Although a direct cause and effect relationship has yet to be demonstrated between MTV-2 activation and T-cell lymphoma, our results, when combined with the data of Michalides et al. (15) and those of Dudley and Risser (5) on MuMTV amplification in C57BL lymphomas, suggest the possibility that hypomethylated proviruses are more prone to escape the transcriptional controls which render them inactive. If this speculation has any degree of validity, we might then expect to encounter active MTV-2 proviruses in some diseases of male GR/A mouse testes in which the MTV-2 provirus also is hypomethylated (Fig. 2D).

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