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The Bulk Chromatin Structure of a Murine Transgene Does Not Vary with Its Transcriptional or DNA Methylation Status

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The DNA methylation status of HRD, a murine transgene, can be controlled by the genetic background upon which it is carried. We found the transgene to be transcribed in competent tissues only when undermethylated. Chromatin structure over the transgene was assayed by nuclear accessibility with DNase I, *Msp*I, and *Pst*I. While the transgene was up to fivefold more resistant to *Msp*I when methylated than when not methylated, we observed no such difference with DNase I or *Pst*I. We suggest that methyl-CpG-binding proteins are responsible for the difference observed with *Msp*I, but that the chromatin structures are otherwise similarly compacted. Methylation could, therefore, play a regulatory role in gene expression beyond that which can be accomplished by bulk chromatin structure alone.

Cytosine methylation occurs over approximately 60% of the CpG dinucleotides in the vertebrate genome (4) and has long been associated with a repression of gene activity (9). The pattern of methylation across the genome is passed to subsequent cell generations by the maintenance methylation activity of the methyltransferase enzyme (5, 18). The importance of DNA methylation in the mammalian genome has been demonstrated by the inability of mice homozygous for a targeted disruption of the methyltransferase gene to complete development (26).

The mechanism by which DNA methylation effects gene repression remains unclear. Several lines of evidence suggest that methylation may interfere with gene expression directly. The cytosine analog 5-azacytidine is capable of reactivating silent genes, presumably by demethylating CpGs which reside in regulatory regions (22). Also, certain transcriptional activator proteins whose cognate sequences contain CpGs have been demonstrated to bind only when the CpG is not methylated (31, 41). These cases of methylation-sensitive binding were determined in vitro, however; genomic footprinting of the tyrosine aminotransferase promoter demonstrated that binding of the CREB transcription factor could not be induced by demethylation of its binding site, even though it exhibited methylation-sensitive binding when assayed in vitro (42). Furthermore, some transcription factors bind their targets equally well in vitro, whether methylated or not (19).

Other lines of evidence suggest that the repressive effect of methylation is indirect and mediated by chromatin structure. Although in vitro methylation of DNA constructs prior to transient transfection renders them transcriptionally inert, the onset of repression after transfection is delayed and correlates with the formation of chromatin over the methylated DNA (7). Similarly, V(D)J recombination of extrachromosomal substrates is inhibited by in vitro CpG methylation, but only after replication (20). As the acquisition of resistance to exogenous endonucleases also occurs after replication, the inhibition of V(D)J recombination is presumably the result of the formation of chromatin over the extrachromosomal substrate rather than the presence of the methyl moieties. Other work has shown that only portions of the transfected DNA need to be methylated to impart transcriptional repression or nuclease resistance over the entire construct, suggesting a spreading of chromatin from the methylated CpGs (23). The chromatin which forms preferentially over methylated substrates may include or even be initiated by proteins which specifically bind methyl CpGs (1, 6, 25, 30).

One difficulty in interpreting these results is that the experimental systems require the analysis of function in vitro or the introduction of cloned DNA into the cell. The chromatin structure over several endogenous genes has been examined in relation to transcription and methylation. The general consensus from these studies is that transcriptionally active genes are undermethylated and reside in an accessible chromatin structure. Study of most of these loci relied upon the inducibility of expression by hormone treatment or by differentiation, while others used differential allelic expression (3, 33). In the present study, we have used a murine transgene whose methylation can be controlled by genetic background (13). Briefly, the HRD transgene is highly methylated when carried in the C57BL/6 (B6) inbred strain and almost completely unmethylated when carried in the DBA/2 (D2) inbred strain. A single locus, *Ssm*1 (for strain-specific modifier), which controls the methylation status of the transgene was identified on distal chromosome 4. Because we are able to control the methylation status of the transgene, it is possible to examine the functional consequences which methylation may have on gene expression and accessibility.

MATERIALS AND METHODS

Transgenic mice. The HRD transgenic construct has been described previously (14). It consists (5′ to 3′) of the mouse immunoglobulin (Ig) heavy-chain enhancer, metallothionein-1 promoter, Ig rearrangement signal sequences from Vκ and Jκ gene segments flanking the rat preproinsulin translation initiation codon, *Escherichia coli* xanthine-guanine phosphoribosyltransferase (*gpt*) coding region, and simian virus 40 splice and poly(A) addition signals. The particular transgenic mouse line, 342, carries seven head-to-tail tandem copies of the HRD construct integrated at an unidentified chromosomal location (15). This line was used exclusively for all experiments described here.

RNA sample preparation and RNase protection assays. Tissue RNAs were prepared by the guanidinium-cesium chloride centrifugation method (27). RNase protection assays were performed essentially as described previously (16). For each sample, 20 μg of total RNA was hybridized simultaneously with three α-^{32}P-labeled antisense RNA probes for *E. coli gpt*, the mouse Ig kappa light-chain constant region (Cκ), and the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Cadmium induction. Transgenic male mice, 19 to 20 weeks of age, were injected with 57 mg of $3CdSO_4 \cdot 8H_2O$ per kg of body weight (25 mg of Cd per

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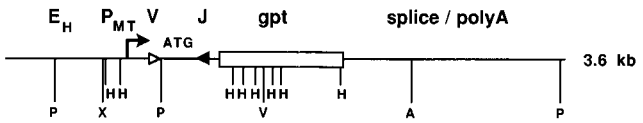

FIG. 1. Map of a single HRD transgene copy. E_H, mouse Ig heavy-chain enhancer; P_{MT}, mouse metallothionein-1 promoter; V, Vκ gene segment; ATG, initiation codon from the rat preproinsulin gene; J, Jκ gene segment; gpt, *E. coli gpt* coding region; splice/polyA, simian virus 40 early region splice and polyadenylation signals; H, *Hpa*II-*Msp*I; P, *Pst*I; X, *Xba*I; V, *Eco*RV; A, *Ase*I.

kg) subcutaneously. Animals were sacrificed 3 h later, and various organs were harvested for isolation of DNA and/or RNA.

DNA probes. The 2.2-kb *Eco*RI-*Xba*I fragment spanning exons 1 and 2 of the mouse dihydrofolate reductase (DHFR) gene was isolated from a genomic clone (DHFR 5′ R34, corresponding to pDR34 [10]) generously provided by R. Schimke (Stanford University) and subcloned into *Eco*RI-*Xba*I-digested pKS$^+$ (Stratagene) to give pKSdhfrRX. The DHFR 5′ region probe was isolated as a 2.2-kb *Kpn*I-*Sac*I fragment from pKSdhfrRX. The transgene-specific probe, gptSV, was isolated as a 2.1-kb *Kpn*I-*Pst*I fragment from pHRD (14). For analysis of the left (5′-most) and right (3′-most) copies of the transgene array, a 0.8-kb *Bam*HI-*Apa*I fragment containing the entire coding region of *gpt* was used as a hybridization probe. All were labeled by a random primed reaction (Boehringer Mannheim) with [α-^{32}P]dCTP (NEN/DuPont).

RNA probes. The antisense riboprobe for *gpt* was generated from a subclone of pHRD, for Cκ from Cκ-2 (35), and for GAPDH from a cloned PCR product amplified from a mouse pre-B-cell library (36) with primers generously supplied by N. Hay (University of Chicago). The Vκ167 (Ig κ light-chain variable region gene segment utilized by the MOPC-167 myeloma) riboprobe was generated from pGEMVκ167 (38). All were labeled by in vitro transcription (Stratagene) with either T3 or T7 RNA polymerase and [α-^{32}P]CTP (NEN/DuPont).

Isolation of nuclei. Nuclei were prepared from freshly dissected mouse kidney, liver, and spleen by ultracentrifugation through a sucrose cushion (28). Aliquots were frozen in liquid nitrogen and stored at −80°C for later use.

Nuclear accessibility assays. DNase I sensitivity analyses were performed by digesting approximately 6×10^6 spleen nuclei or 1.5×10^6 kidney nuclei with 0, 5, 10, 20, 50, or 100 U of RQ1 DNase (Promega) per ml for 2 min or with 100 U/ml for 8 min at 37°C. DNase I reactions were performed in a 50- to 55-μl volume of 45 mM Tris-Cl (pH 8)–10 mM NaCl–6 mM $MgCl_2$–1 mM $CaCl_2$. *Msp*I and *Pst*I sensitivity analyses were performed by digesting approximately 3×10^6 nuclei with 0, 5, 10, 50, or 100 U of restriction enzymes *Msp*I and *Pst*I (New England Biolabs), respectively. Digest reactions were performed in a 50-μl volume according to the manufacturer's specifications, except with the addition of 0.1 mM EGTA, and incubated at 37°C for 1 h. All digestions were terminated by addition of an equal volume of 20 mM EDTA–1% sodium dodecyl sulfate–1 mg of proteinase K per ml, and the mixtures were incubated overnight at 37°C. DNA was prepared by organic extraction and ethanol precipitation.

Southern analysis. DNA samples were restricted with various restriction enzymes according to the manufacturer's specifications and electrophoresed through 1% SeaKem GTG agarose gels (FMC) in 1× TAE or TBE (37). The contour-clamped homogeneous electric field gel was 1% SeaKem GTG in 0.5× TBE and was run for 12 h at 200 V at 14°C with switching times ramped from 1.0 to 3.0 s, using the CHEF-DRII apparatus (Bio-Rad). All gels were transferred to Hybond-N$^+$ nylon membranes (Amersham), hybridized with α-^{32}P-labeled probes, and autoradiographed. Cronex Lightning-Plus (DuPont) intensifying screens were used as indicated in each figure legend. Blots which were hybridized with multiple probes were stripped between successive hybridizations according to the manufacturer's specifications.

RESULTS

Transcription of the HRD transgene depends on its methylation status and the tissue specificity of its promoter/enhancer. Previous results showed that deletional rearrangement of the transgene (diagrammed in Fig. 1) could be found primarily within undermethylated copies (13, 15) (see Fig. 4, top right). Although the methylation state as assayed in the adult spleen could have changed since the rearrangement event, the correlation suggests that undermethylation may be indicative of locus accessibility, in particular to the V(D)J recombinase. As transcription may also be taken as a measure of locus accessibility, we looked for transgenic transcripts in adult tissues. Northern (RNA) analysis of 20 μg of total RNA failed to demonstrate any expression from either methylated or unmethylated transgenes (data not shown). Using the more sensitive RNase protection assay, however, we were able to detect transcripts in certain tissues of mice which carried the transgene either unmethylated or partially undermethylated. Little to no expression was detected in tissues where the transgene was highly methylated (Fig. 2).

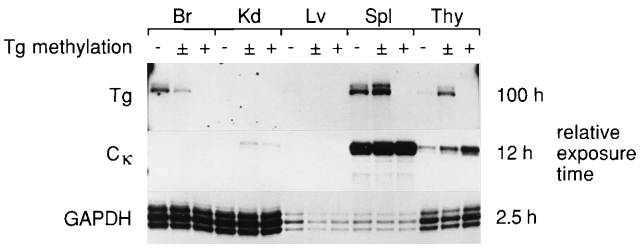

FIG. 2. Expression of the HRD transgene varies with methylation. RNase protection assay of various tissue RNAs from transgenic mice with different HRD methylation states (+, completely methylated; ±, partially methylated; −, completely unmethylated) was performed. Protected species only are depicted and are indicated at the left (Tg, *gpt* from the HRD transgene). Relative exposure times were calculated to account for decay of the radioactive isotope and are indicated at the right. Br, brain; Kd, kidney; Lv, liver; Spl, spleen; Thy, thymus.

Complete or partial undermethylation of the transgene is clearly not sufficient for expression; only tissues in which the enhancer/promoter are active can generate transcripts. Spleen and brain yielded transcripts when the transgene was unmethylated or partially undermethylated, but kidney and liver did not (Fig. 2). Partial methylation occurs as the methylated transgene in the B6 background is crossed into the D2 background (13) and probably results from inheritance of a methylated transgene into a background with two D2 alleles of *Ssm*1 (43). The signal in the thymus sample is probably due to contaminating B cells from nearby hilar lymph nodes taken during dissection, as evidenced by the presence of B-cell-specific Cκ transcripts. The expression in brain is expected, given the activity of metallothionein constructs in brain (32), and probably genuine, as Cκ transcripts are absent. These expression patterns suggest that undermethylation of the transgene is attended by a state of accessibility (albeit transient; see Discussion) to the transcriptional machinery and to ambient nuclear factors.

Transgene expression can be induced by treatment with heavy metals. As four of five identified metal regulatory elements from the endogenous metallothionein-1 promoter were retained in the transgene construct (39), we attempted to induce expression by treatment with heavy metals. We administered cadmium to mice carrying either methylated or unmethylated transgenes and examined expression by RNase protection (Fig. 3). The enhancement of expression by heavy-metal treatment is clear only in unmethylated transgenic kidney and liver. This is understandable in that kidney and liver are the sites of highest heavy-metal accumulation in the body (11). Despite contamination of the kidney sample with peripheral B cells, as indicated by the presence of Cκ transcripts, the induction is probably significant (compare the transgene/Cκ ratio with that in Fig. 2) and not due to induction of the contaminating B cells (compare the induced kidney transgene/Cκ ratio with that in spleen). It is difficult to assess whether heavy-metal treatment induced expression from methylated transgenes, as uninduced methylated samples were not run in parallel. Furthermore, it is not at all clear that the faint bands indicating transcription from induced methylated samples (kidney and spleen) arose from methylated copies of the transgene: rare undermethylated copies can be detected in methylated samples by extended exposure of *Hpa*II-digested genomic Southern blots (data not

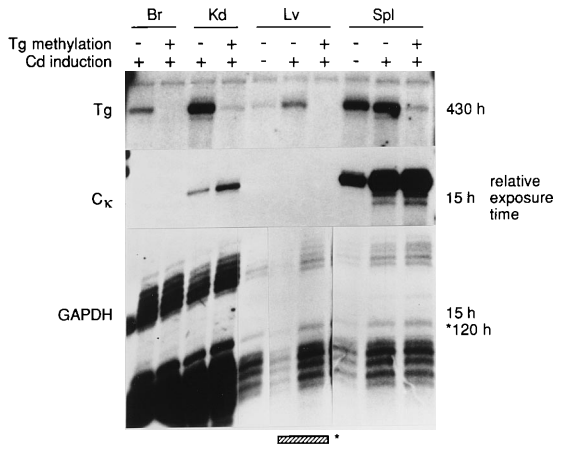

FIG. 3. Expression of the HRD transgene is heavy-metal inducible. RNase protection assay of various tissue RNAs from transgenic mice with and without Cd^{2+} induction. Labels are as described for Fig. 2. The GAPDH signal in Cd^{2+}-treated livers required extended exposure and is indicated by the hatched bar.

shown). Nonetheless, transcription is generally inhibited by the presence of methylation over the transgene.

DNase I does not reveal differences in locus accessibility between the methylated and unmethylated transgenes. As the HRD transgene was rearranged and transcribed only when unmethylated or partially undermethylated, we wanted to see if we could demonstrate a difference in locus accessibility directly. To this end, nuclei were isolated from various organs of mice carrying either methylated or unmethylated transgenes. These nuclei were treated with DNase I with the intention that DNA loci packaged in compacted chromatin would be more resistant to digestion (33, 34). After purifying genomic DNA from the DNase I-treated nuclei, we analyzed the samples by restriction digestion and Southern blotting.

The extents survival of the transgene band (Tg in Fig. 4) through increasing DNase I concentrations are highly similar whether the transgene is methylated or not in both kidney and spleen (Fig. 4, top). To control for variations between nuclei preparations, DNase I digestions, and sample loadings, the same blots were rehybridized with a probe for a constitutively active gene, DHFR (Fig. 4, bottom). Clearly, the DNase I digestion profiles between sample sets are also highly similar for the DHFR locus. The extra series of transgene bands (TgΔ) in the unmethylated spleen samples corresponds to copies of the transgene which have undergone V(D)J deletional rearrangement. Importantly, its pattern of DNase I sensitivity does not differ from the unrearranged series of bands (Tg) either from the same spleen or from the rearrangement-incompetent kidney. The similarity of these DNase I digestion profiles suggests that the gross chromatin structure over the transgene does not vary significantly between expressing and nonexpressing tissues (spleen versus kidney), rearranged and nonrearranged copies (spleen, TgΔ versus Tg), or methylated and unmethylated versions.

The persistence of the transgene band through increasing DNase I compared with the rapid disappearance of the DHFR band (Fig. 4) suggests that the transgene is packaged in condensed chromatin in both methylated and unmethylated tissues. This observation is not an artifact of differing target sizes (larger bands are presumably more sensitive to digestion since they present larger targets for the enzyme). The transgene is at least 10 times more resistant to DNase I than is DHFR (compare band intensity ratios, i.e., 50 to 0 U of DNase I for transgene and 5 to 0 U of DNase I for DHFR), while the difference in target sizes is less than twofold (6.5-kb DHFR band versus 3.6-kb transgene band). Furthermore, the 3′-most copy in the array hybridized at 6.6 kb (larger than the DHFR band) as a result of the integration site and demonstrated the same resistance pattern as the 3.6-kb band (data not shown). Similar blots were rehybridized with a probe for the immunoglobulin light-chain variable region gene segment, Vκ167: the full-length 6.6-kb band (larger than both the transgene and DHFR bands in those blots) persisted through high concentrations of DNase I in a manner similar to that of the transgene (data not shown). As this particular variable region gene segment is not utilized in a significant fraction of total splenic B cells, it is effectively a nonexpressed locus in spleen (and kidney). The greater similarity of the transgene to the silent Vκ167 locus than to the constitutive DHFR locus with respect to DNase I sensitivity argues strongly that the transgene is packaged in condensed, inactive chromatin.

***Msp*I treatment reveals differences in locus accessibility between methylated and unmethylated transgenes.** We also probed chromatin structure with a different enzyme, *Msp*I, which cleaves the site CCGG, regardless of methylation of the

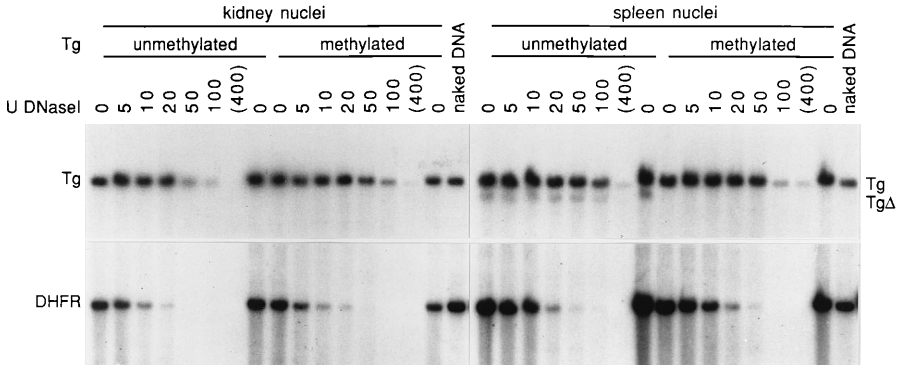

FIG. 4. Transgenic chromatin is equivalently accessible to DNase I, regardless of DNA methylation. Kidney and spleen nuclei were treated with the indicated concentrations of DNase I. DNA was prepared, digested with *Eco*RV, Southern blotted, and hybridized with the indicated probes. The top panel shows bands of 3.6 kb in size hybridizing with a transgene-specific probe (Tg). The bottom panel shows bands of 6.5 kb in size hybridizing with endogenous control probe (DHFR). Naked DNA sample serves as a hybridization standard between probes. Unrearranged (Tg) and rearranged (TgΔ) transgene bands of 3.6 and 3.3 kb in size, respectively, in spleen are indicated at the right. Autoradiography was performed without intensifying screens.

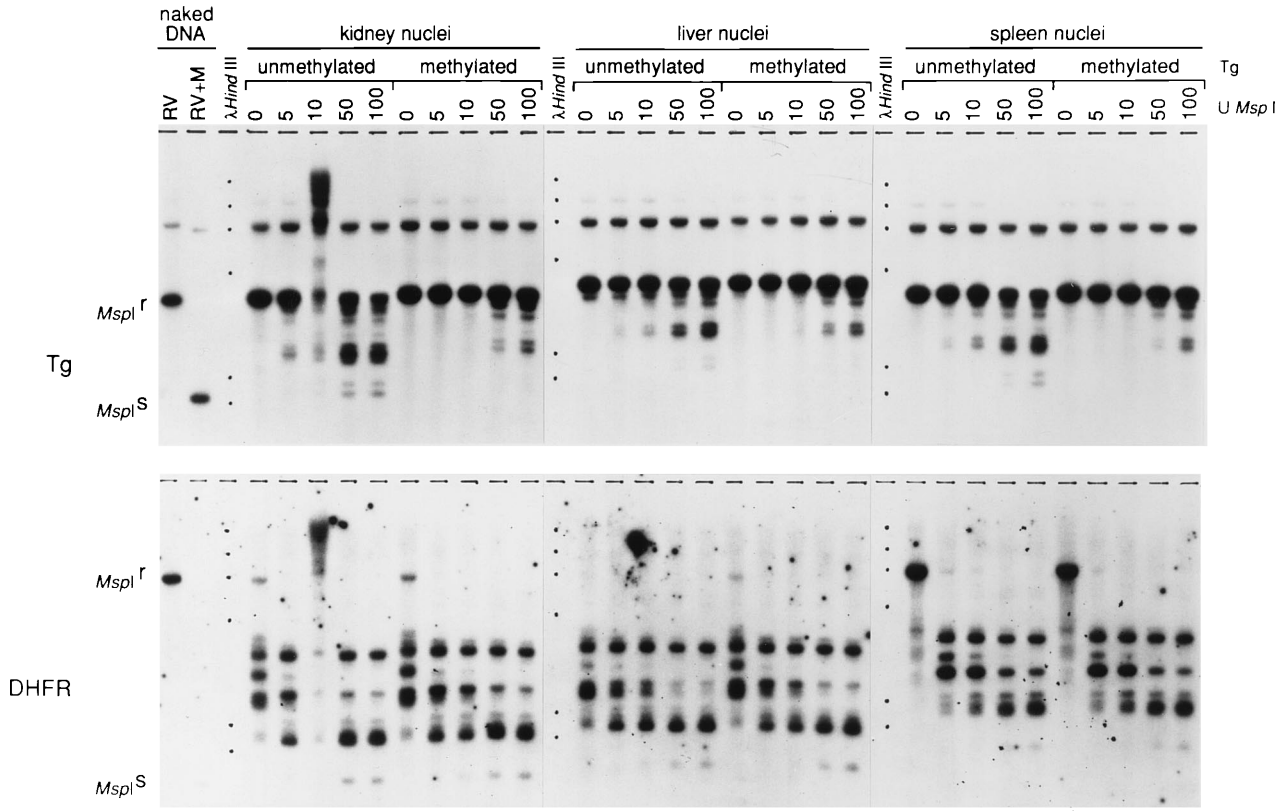

FIG. 5. Transgenic chromatin is less accessible to *Msp*I when DNA is methylated. Nuclei were treated with the indicated amounts of *Msp*I (units per reaction) for 1 h. DNA was prepared, digested with *Eco*RV, and Southern blotted. Naked DNA samples were digested with *Eco*RV alone (RV) or with *Eco*RV plus *Msp*I (RV+M) to demonstrate bands indicative of complete resistance (*Msp*I^r) and sensitivity (*Msp*I^s) to *Msp*I, respectively. The top panel was hybridized with a transgene-specific probe (Tg); the bottom panel was probed with an endogenous control probe (DHFR). λ*Hin*dIII marker positions are indicated at 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb. Autoradiography was performed with intensifying screens.

internal cytosine. Kidney, liver, and spleen nuclei from mice carrying either methylated or unmethylated transgenes were treated with increasing concentrations of *Msp*I. After termination of the *Msp*I digestion, DNA was isolated and subjected to restriction digestion and Southern blot analysis. The blot was hybridized with a transgene-specific probe (Fig. 5, top) and with the 5′ region of DHFR (Fig. 5, bottom). Bands corresponding to complete resistance and sensitivity to *Msp*I are indicated. Note that bands whose intensity changes with increasing *Msp*I are most informative for assessing *Msp*I accessibility. The unmethylated transgenic kidney sample treated with 10 U of *Msp*I apparently did not cut to completion with the second enzyme, *Eco*RV. Each of the organs demonstrated enhanced resistance to *Msp*I digestion when the transgene was methylated: approximately 10-fold for kidney and liver but only 2-fold for spleen. This difference was transgene specific, as the DHFR control demonstrated.

In consideration of the possibility that the observed differences were due to the ability of *Msp*I to cleave unmethylated DNA more efficiently than methylated DNA, naked genomic DNA from methylated and unmethylated transgenic liver was digested with increasing amounts of *Msp*I under conditions nearly identical to those used with the nuclei. We observed *Msp*I to cleave unmethylated sequences approximately twice as efficiently as methylated sequences (data not shown), in close agreement with the results of Butkus et al. (8). Thus, when the twofold difference in *Msp*I cleavage rate is taken into account, the differences attributable to nuclear accessibility are reduced to fivefold for kidney and liver and negligible for spleen.

***Pst*I treatment does not reveal differences in locus accessibility between methylated and unmethylated transgenes.** As the *Msp*I result for kidney and liver was difficult to reconcile with the DNase I result, we considered that methyl-CpG-binding proteins (MeCPs), as described by Bird's laboratory (25, 30), might be involved. In that event, the *Msp*I difference could simply be a result of MeCPs physically interfering with access to methylated CpGs at *Msp*I sites in the transgene, rather than to gross alterations in chromatin structure. To test this possibility, we repeated the experiment, except using *Pst*I (whose site does not contain a CpG) to treat the nuclei (Fig. 6). Bands corresponding to complete resistance and sensitivity to *Pst*I are indicated. Again, bands whose intensity changes with increasing *Pst*I are most informative for assessing *Pst*I accessibility. The unmethylated transgenic kidney sample treated with 10 U of *Pst*I apparently did not cut to completion with the second enzyme, *Eco*RV. The methylated and unmethylated transgenes demonstrated highly similar *Pst*I digestion profiles in kidney, liver, and spleen (Fig. 6). The ability of *Msp*I, but not DNase I or *Pst*I, to reveal differences in nuclear accessibility suggests that CpG targets are less accessible when methylated but that the overall chromatin structures are similar whether the transgene locus is methylated or not. We suggest that MeCPs may be responsible for mediating the protection of methyl-CpGs from *Msp*I.

The presence of bands other than the completely resistant species in the samples with no added *Msp*I or *Pst*I is probably due to digestion by endogenous nucleases. This idea is supported by the fact that many of these bands do not change in

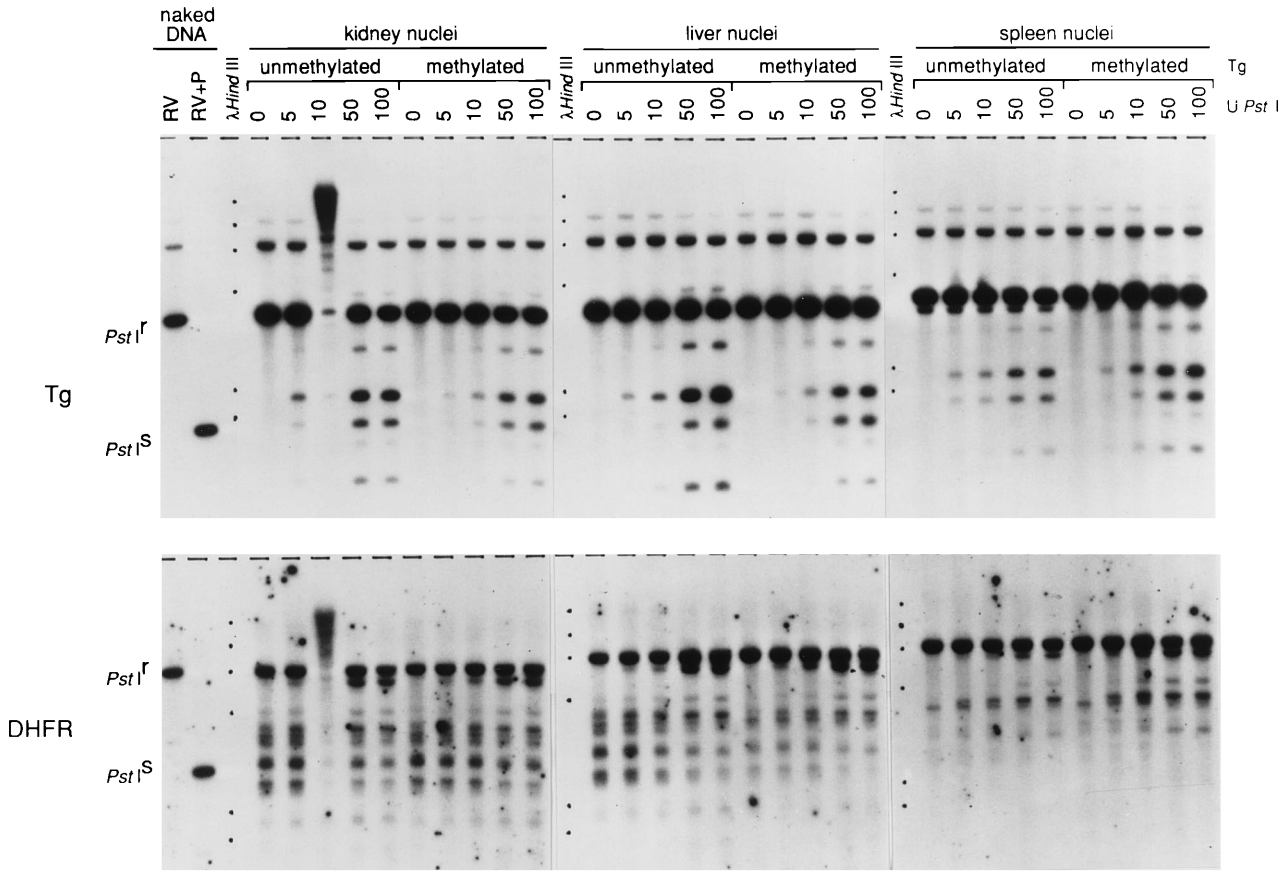

FIG. 6. Transgenic chromatin is equivalently accessible to *Pst*I, regardless of DNA methylation. The assay was performed as described for Fig. 5 except with *Pst*I (P) in place of *Msp*I.

intensity with increasing *Msp*I or *Pst*I. Interestingly, these bands are much more apparent upon hybridization with the DHFR probe than with the transgene probe. This may be taken as added support for the transgene being packaged in closed, inactive chromatin.

Chromatin structure is homogeneous across the transgene locus. As the expression level that we observed from the unmethylated transgene in spleen was rather low, it may be that only one or a few of the seven copies in the array were actually generating transcripts. Since it is impossible to determine from which of the copies the transcripts emanated, it is crucial to demonstrate homogeneity of chromatin structure over the entire array if any conclusions are to be drawn relating expression to the DNase I, *Msp*I, and *Pst*I sensitivities as determined above. The analyses presented in Fig. 4 to 6 involved reducing six of the seven copies of the transgene into the same-size (3.6-kb) restriction fragment and assessing of the survival of that fragment through increasing amounts of exogenously supplied endonucleases (the seventh copy hybridized at 6.6 kb as a result of the 3′ integration site). As *Msp*I and *Pst*I sensitivity of the transgenic chromatin was measured by the appearance of new, smaller bands, accessibility of any one of the six copies present in the 3.6-kb band ought to have been detectable. The DNase I sensitivity analysis, however, relied upon the rate of disappearance only of the 3.6-kb band to gauge accessibility: a single accessible copy among five inaccessible copies may have been difficult to detect. To determine if such a lone accessible copy did exist within the array, we analyzed DNase I sensitivity over certain individual copies and over the locus as a whole. We performed this analysis in spleen, since it expresses the transgene and would be most likely to harbor accessible copies if they did indeed exist.

As the 5′-most and 3′-most copies are unique by virtue of their appostition to integration site DNA, we were able to assess their DNase I sensitivities separately from the rest of the array. Both the 5′-most and 3′-most copies demonstrated DNase I digestion profiles which were indistinguishable from those of the rest of the array and of their oppositely methylated counterparts (data not shown). Any uniquely accessible copies must therefore reside in the middle of the array at positions 2 to 6 (Fig. 7a).

Chromatin over the locus as a whole was examined by digesting DNA from DNase I-treated (Fig. 7b) and *Msp* I-treated (Fig. 7c) spleen nuclei with a restriction enzyme (*Eco*RI) which releases the entire seven-copy array plus 3 kb of integration site DNA as a 30-kb band (Fig. 7a). If any one transgene copy within the array were DNase I sensitive or *Msp*I sensitive while the other six were not, unique digestion products of predictable size ought to be discernible. For instance, if copy 3 (5′ to 3′) were the only accessible copy, then DNase I or *Msp*I digestion ought to convert the 30-kb band into two bands of ~9.5 and ~17 kb (Fig. 7a). Potential digestion products for sole accessibility of any one of the other six copies can similarly be predicted. No such bands, however, could be detected (Fig. 7b [top] and c), suggesting that chromatin structure is homogeneous across the array. The DNase I blot was stripped and

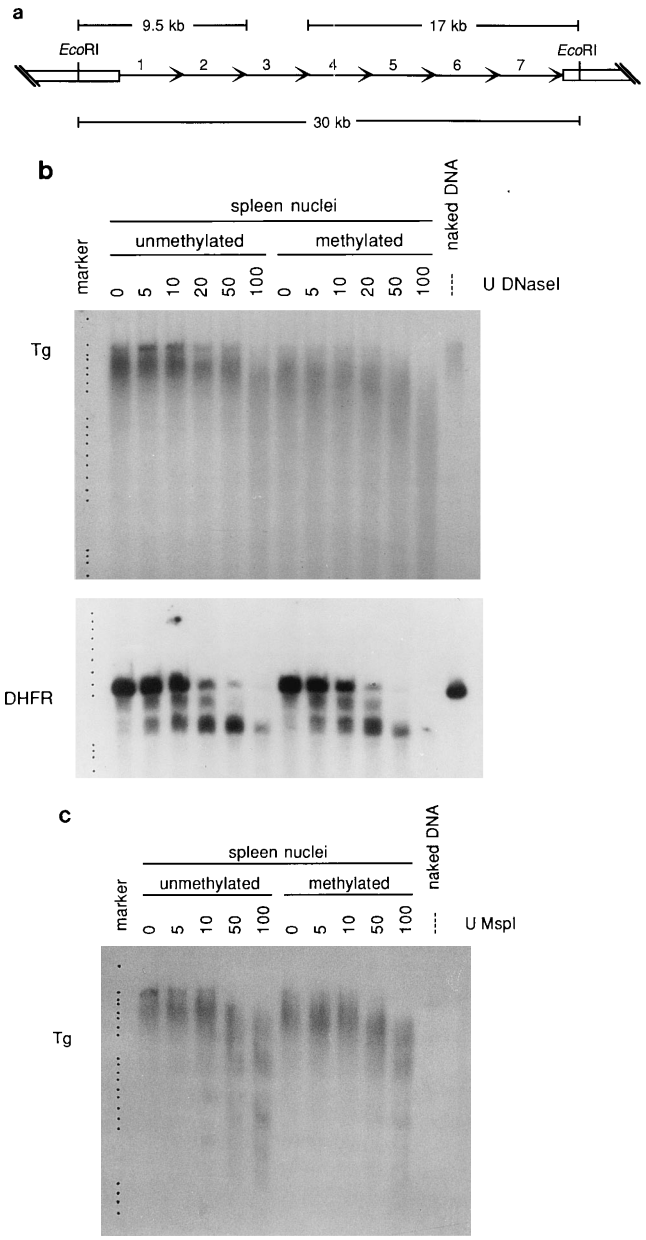

FIG. 7. Transgene demonstrates homogeneous chromatin structure across the integration locus. (a) Map of seven-copy transgene locus. Predicted digestion products of 9.5 and 17 kb are indicated for sole accessibility of copy 3. Spleen nuclei were treated with the indicated concentrations of DNase I (b) or *Msp*I (c). DNA was prepared, digested with *Eco*RI, Southern blotted, and hybridized with the probes indicated at the left. Marker lane contains λ restriction fragments (48.5, 33.5, 29.9, 27.6, 23.1, 21.2, 17.0, 15.0, 9.4, 8.4, 7.4, 6.5, 5.8/5.6, 4.9, 4.3, 3.8, 3.5, 2.4, 2.3, 2.2, and 2.0 kb [top] and 9.4, 8.4, 7.4, 6.5, 5.8/5.6, 4.9, 4.3, 3.8, 3.5, 2.4, 2.3, 2.2, and 2.0 kb [bottom]). Autoradiography was performed with (DHFR) and without (transgene [Tg]) intensifying screens.

rehybridized with a DHFR probe (Fig. 7b, bottom) to demonstrate that hypersensitive bands could indeed be detected within these samples and to verify equivalent digestion and loading of samples.

The bands which did appear with increasing *Msp*I in Fig. 7c have sizes which are consistent neither with the same single copy nor with a different single copy being accessible in each cell. If a different single copy were accessible in each cell, then

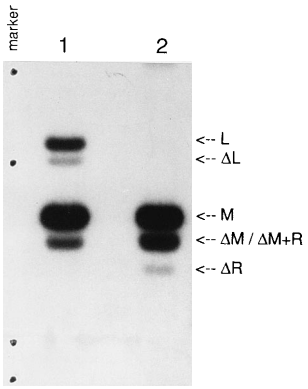

FIG. 8. V-J joining in left (L), middle (M), and right (R) copies in the HRD transgene array. Spleen DNA from a mouse with an unmethylated transgene locus was digested with *Ase*I and *Eco*RI (lane 1) or *Xba*I and *Eco*RI (lane 2), Southern blotted, and hybridized with a transgene-specific probe. Deletion products (Δ) resulting from V-J joining within the various copies of the array are indicated. The middle deletion copies and right copy (ΔM+R) comigrate in lane 2. Marker lane contains λ DNA cut with *Hin*dIII (6.6, 4.4, 2.3, and 2.0 kb).

one would expect to see series of bands which differ by multiples of the unit transgene length (3.6 kb). This was not the case, however; the bands are much more closely spaced and probably represent partial *Msp*I digestion products. These bands appeared in the unmethylated transgene at levels of *Msp*I approximately twofold lower than that for the methylated transgene. As described earlier, this difference can be attributed to the lower cleavage rate *Msp*I demonstrates for methylated targets. Importantly, these bands appeared for both the unmethylated and methylated transgenes; therefore, even if they did represent lone accessible copies within the array, they could not possibly explain the expression from only the unmethylated transgene.

Further support for the contention that chromatin structure is homogeneous across the seven-copy array is given by the finding that the left (copy 1), middle five (copies 2 to 6), and right (copy 7) copies were all accessible to the V(D)J recombinase when unmethylated in spleen. Figure 8 shows deletion products resulting from V-J joining present for each of the left, middle five, and right copies. Although the band intensities were not quantitated, the ratios of the rearranged (ΔL, ΔM, and ΔR in Fig. 8) to unrearranged (L, M, and R) bands appear to be similar, suggesting similar frequencies of recombination and, presumably, similar levels of accessibility. As the middle five copies cannot be distinguished individually, it is possible that all the observed V-J joining for the middle five copies had occurred from only one of the five. A lone accessible copy among the middle five, however, would have to be rearranged at a fivefold-higher frequency than either the left or right copies to produce the observed similarity of rearranged to unrearranged band ratios. Little to no V-J joining can be detected when the transgene is methylated (15).

We have assayed chromatin structure physically by nuclear accessibility to exogenous endonucleases as well as functionally by V-J joining and found it to be homogeneous across the entire seven-copy transgene array and compacted much like an inactive locus. The expression which emanates from the seven unmethylated copies in spleen would presumably be homogeneous as well, each copy expressing at a level comparable to that of its neighbors. The suppression of expression from the methylated transgene array would therefore seem to be mediated by the methylation itself rather than by a more compacted chromatin structure.

DISCUSSION

The HRD transgene has previously been found to be subject to strain-specific methylation. This methylation appears to be under the control of a single genetic locus on chromosome 4 near *Fv1*, named *Ssm1* (13). We have demonstrated here that the methylation modification is accompanied by a repression of transcription, but not by a dramatic alteration in chromatin structure, compared with the unmethylated transgene locus. Together with the previous observation that V(D)J recombination does not occur within methylated transgenes (15), these data suggest that methylation is capable of exerting a level of control over gene activity beyond that which can be accomplished by bulk chromatin structure alone. Although the presence of MeCPs in the chromatin fiber may represent a fine structural alteration, the paradigm of active genes residing in open chromatin versus repressed genes residing in closed chromatin would seemingly not hold in this situation.

The use of the terms "active" and "repressed" to describe gene expression and "open" and "closed" to describe chromatin structure raises many issues. For instance, we must consider whether the observed levels of transgene expression (and rearrangement) truly qualify the locus as active. That is, does expression which requires an extended exposure of an RNase protection assay to detect constitute active expression? Does rearrangement, which requires only a single window of accessibility to the V(D)J recombinase in a cell's past lineage, constitute open accessibility? For that matter, it has clearly not been established that chromatin structure can be partitioned into the two states, open and closed. While the relative resistance of the transgene to digestion either by DNase I (Fig. 4) or endogenous nucleases (Fig. 5 and 6; see Results) suggests that it resides in closed chromatin (like that of an inactive locus), the *Msp*I-accessibility difference in kidney and liver suggests that this closed structure can exist in at least two states: that with MeCPs and that without.

We were not able to demonstrate any significant difference attributable to chromatin structure between methylated and unmethylated transgenes in spleen. The lack of a clear indication of MeCPs bound over the methylated transgene in spleen may have been due in part to the greater proportion of cells in the spleen (compared with kidney and liver) which were cycling and, therefore, disassembling their chromatin in order to replicate their genomes. Any bound MeCPs would presumably have been stripped from the methylated DNA in this process. We still believe MeCPs to be bound over the methylated transgene in spleen, however; bands indicating *Msp*I access to the unmethylated transgene appear with as little as 5 U *Msp*I, while they do not appear for the methylated transgene until 50 U of *Msp*I is present (Fig. 5, top). The relative intensities of these bands, however, do not clearly establish a difference dramatically greater than twofold, a difference attributable to *Msp*I cleavage rates for unmethylated over methylated naked DNA.

The observed level of expression and rearrangement of the unmethylated transgene in spleen occurred from a locus which we have argued to reside in a relatively compacted chromatin state. This level of expression and rearrangement may represent either a programmed active state or leakiness from an intended inactive state. An extensive amount of work, however, has established the paradigm that active genes reside in open chromatin. This has been demonstrated for many endogenous loci (12, 17, 29, 40) and also has basis in a history of cytogenetic evidence (2, 24). Therefore, we favor the interpretation that the observed level of expression and rearrangement from the unmethylated transgene represents leak-through rather than bona fide activity. The activity from the locus may occur during periods of chromatin "breathing." If one views chromatin not as a static condition but as a dynamic process of assembly and disassembly of a complex multiprotein structure, then it might seem entirely reasonable to expect RNA polymerase [or V(D)J recombinase] to make a few passes over a locus on occasion. In that event, it appears that the function of methylation would be to prevent leaky expression and accessibility, presumably by stabilizing the assembled state to a greater degree over the disassembled state. Such a role could well be mediated by protein-protein interactions between MeCPs and chromatin structural proteins. This idea is consistent with a popular opinion that methylation plays a secondary role in the regulation of gene expression and yet also with the dramatic demonstration that this role is an essential one (26).

Although transcription, open chromatin, and undermethylation appear to be related by frequent association, their causal relationships remain unclear. Our results indicate that undermethylation of a locus is not sufficient to define an open and accessible chromatin configuration and, conversely, that packaging of a locus into compacted chromatin is not sufficient to target it for methylation. Although methylation would presumably direct the binding of MeCPs to the transgene, it cannot be pivotal in defining the overall level of chromatin compaction over the locus. It may be simply that the absence of high-level transcription, regardless of methylation, allows for compaction of chromatin over the locus. Experiments with the Ig μ enhancer core (also present in our transgene), however, suggest that an accessible state can be maintained in the absence of transcription (21). While we have argued that the observed expression of our transgene represents leaky transcription, it is clear that undermethylation is necessary (though not sufficient) for the transcription that does occur, and that methylation can effectively suppress that leakiness.

Our results suggest that the role of methylation in the genome may be to strengthen repression at a locus by recruiting MeCPs into the overlying chromatin. These proteins may stabilize the chromatin structure, thereby effecting tighter repression. The question remains, however, as to what directs methyltransferase to methylate some loci but not others. As methylation of the HRD transgene is controlled by the *Ssm1* locus, it will be interesting to determine the nature of the presumed gene product of the *Ssm1* locus and the role that it may play in gene regulation.

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