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Demethylation and expression of murine mammary tumor proviruses in mouse thymoma cell lines

(dexamethasone/DNA methylation)

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ABSTRACT **Murine mammary tumor virus (MMTV) expression is analyzed in a T-lymphoid cell line (T_1M_1) sensitive to the killing effect of glucocorticoids and in two of its variants, one resistant ($T_1M_1^r$) and one supersensitive ($T_1M_1^{ss}$) to glucocorticoid-induced lymphocytolysis. In the T_1M_1 line, MMTV is expressed and induced approximately 10-fold by short treatment with dexamethasone. Southern blot analyses of restriction enzyme digests of DNA from T_1M_1 cells reveal three proviruses similar to those of normal C57BL mouse tissue. In the $T_1M_1^{ss}$ line, which has retained functional glucocorticoid receptors, MMTV mRNA is inducible by glucocorticoids, while induetion is reduced in the $T_1M_1^r$ line defective in glucocorticoid receptors. Moreover, the $T_1M_1^r$ line expresses a strikingly elevated basal level of MMTV mRNA in the absence of hormone. No rearrangements or superinfection have occurred in the variants, but all the regions containing 5′-long terminal repeats are demethylated in the $T_1M_1^r$ variant although other sites of the provirus remain methylated. Because this variant was selected by prolonged treatment with dexamethasone, these observations raise the possibility that the continuous transcription of MMTV that occurred during this selection can result in glucocorticoid-induced demethylation of long-terminal-repeat sequences.**

Glucocorticoids induce in a variety of tissues specific responses by mechanisms that appear to be similar to those of other steroid hormones. Glucocorticoids induce cytolysis of some murine cell lines derived from malignant lymphoid T cells (1, 2). This response is mediated by the glucocorticoid receptor, which interacts with chromatin, but the mechanism of cell lysis remains unknown. Lymphoid cells allow a genetic approach to study of the mechanism of glucocorticoid hormone action because variants resistant to killing can be selected easily. A number of resistant variants have been isolated and characterized from S49 and W7 cell lines (1–3). More recently, variants supersensitive to glucocorticoid-induced killing have been isolated from W7 and T_1M_1 cell lines (4).

Glucocorticoids also regulate the transcription of integrated murine mammary tumor virus (MMTV) proviral DNA in various cell types (5, 6) including lymphoid cell lines (7). The availability of lymphoid cell variants having different sensitivities to the lytic effect of glucocorticoids allows the study of MMTV expression as a function of glucocorticoid sensitivity. Therefore, we have analyzed the control of MMTV RNA concentration by dexamethasone in wild-type, resistant, and supersensitive T_1M_1 cell lines by using cloned MMTV sequences.

There is evidence that modification of DNA such as methylation may be correlated with changes in gene expression (8), in particular, in MMTV proviral DNA of mouse tissue (9). By using methylation-sensitive restriction enzymes to detect specific methylation of MMTV sequences, we have compared the methylation of sites within or flanking the long terminal repeat (LTR) (10) with that of a site within the MMTV envelope gene.

In this paper, we describe the regulation of MMTV RNA synthesis and the methylation of the MMTV LTR and envelope sequences in sensitive, supersensitive, and resistant thymoma cell lines. We present evidence that the expression of endogenous proviruses is different in the various cell lines and appears to correlate with methylation of the LTR.

MATERIALS AND METHODS

Cell Lines and Growth. T_1M_1 designates the subclone T_1M_1b derived from T_1M_1 4G-1.3, a thymoma from a C57BL/6 mouse obtained from R. Hyman (The Salk Institute). $T_1M_1^{ss}$ designates the dexamethasone-supersensitive clone T.12.15.19 derived from T_1M_1 (4). $T_1M_1^r$ is a dexamethasone-resistant clone, T135^r, derived from T_1M_1 by Ronald Newby (The Salk Institute). All cell lines were grown in suspension as described (2).

Preparation of pBR322–MMTV Recombinant Plasmid DNA. *Escherichia coli* 1106 (803 r_k^- m_k^- (11) was used for transformation. MMTV DNA, a gift from H. Diggelman, was subcloned in plasmid pBR322 DNA by transfecting $CaCl_2$-treated *E. coli* cells. Approximately 10^3 tetracycline-resistant colonies were obtained. These were transferred to agar plates covered with a sterile membrane filter and screened by hybridization with ^{32}P-labeled MMTV cDNA (12). Several positive colonies were isolated and shown to contain the *Pst* I MMTV DNA fragment. Clones were grown preparatively and the recombinant plasmids were purified by centrifugation in ethidium bromide/CsCl density gradients.

Gel Electrophoresis of DNA and Filter Transfer Analysis. High molecular weight cellular DNA was purified by proteinase K digestion and phenol extraction. Restriction enzyme-digested DNA was separated electrophoretically on 1% agarose gels, and transfer of DNA to nitrocellulose filters was carried out by the blotting procedure of Southern (13). Hybridization of nitrocellulose filters was carried out with restriction enzyme-cleaved nick-translated MMTV DNA fragments having a specific activity of $\approx 5 \times 10^7$ cpm/μg.

Isolation and Agarose Gel Electrophoresis of MMTV Poly(A)$^+$RNA. Washed cells were suspended in 50 mM Tris·HCl, pH 7.4/150 mM NaCl/50 mM EDTA/1% $NaDodSO_4$ containing 400 μg of proteinase K per ml. Lysed cells were sheared, incubated for 15 min at room temperature, and extracted with phenol and chloroform. Nucleic acids were precipitated with ethanol and collected by centrifugation.

Purified RNA was heated at 90°C for 3 min and cooled

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Abbreviations: MMTV, murine mammary tumor virus; LTR, long terminal repeat; kb, kilobase(s).

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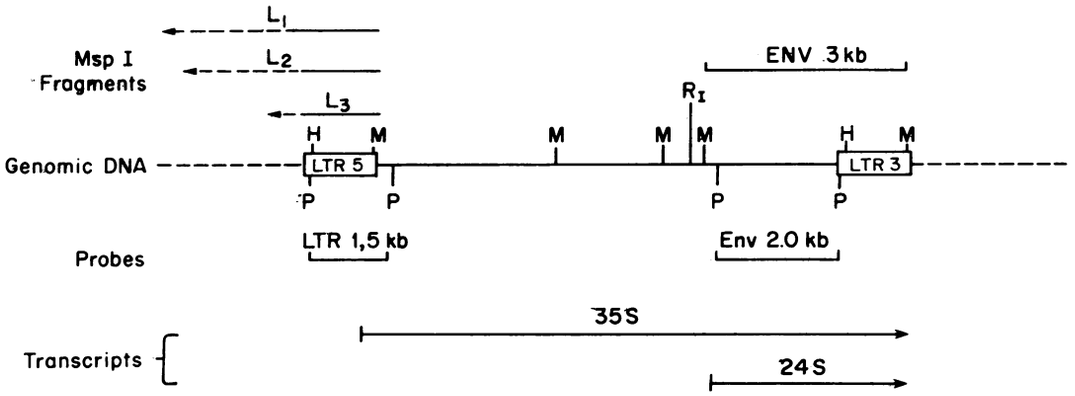

FIG. 1. Restriction map of MMTV provirus. The *Eco*RI (RI), *Msp* I (M), *Pst* I (P), and *Hha* I (H) sites indicated on the map are redrawn from ref. 10. The cloned DNA fragments used as hybridization probes are shown below the map, and the *Msp* I DNA fragments detected by hybridization are shown above the map. The MMTV RNA transcripts are shown below the probes.

quickly, and NaCl was added to 0.5 M. RNA was bound to an oligo(dT)-cellulose column; unbound material was removed by washing the column with binding buffer. Poly(A)$^+$RNA was released from the column by eluting with sterile water and precipitated with ethanol.

Poly(A)$^+$RNA from thymoma cells was denatured with 2 M glyoxal in 10 mM sodium phosphate (pH 6.5) for 1 hr at 50°C and electrophoresed in a horizontal 1.5% agarose gel in 10 mM sodium phosphate (pH 6.5). RNA was transferred to a nitrocellulose filter as described (14). The filter was hybridized for 18 hr at 42°C in 50% formamide/10% dextran sulfate with restriction enzyme-cleaved nick-translated MMTV DNA fragments (specific activity, $\approx 5 \times 10^7$ cpm/μg) (15).

RESULTS

MMTV DNA in T_1M_1 Cell Lines. To compare MMTV proviral DNA in the T_1M_1 cell line and its two variants, we have used the Southern blot technique to analyze cellular DNA cut with the restriction enzyme *Eco*RI. This enzyme has only one cutting site in the MMTV genome (Fig. 1) and gives a MMTV restriction pattern characteristic of each mouse strain (16). Fig. 2 shows that, by hybridizing with a cloned LTR DNA probe, we found that the T_1M_1 line has five DNA restriction fragments characteristic of the C57BL/6 mouse (17). The resistant ($T_1M_1^r$) and supersensitive ($T_1M_1^{ss}$) variants show exactly the same *Eco*RI restriction DNA fragments, which excludes the possibility that gene rearrangements or superinfection might have occurred in these variants.

MMTV Expression in Wild-Type, Supersensitive, and Resistant T_1M_1 Cell Lines. Cells were cultured in the absence or presence of 0.1 μM dexamethasone for 6 hr and RNAs were extracted. RNAs were analyzed on a denaturing agarose gel and transferred to nitrocellulose filters, and the immobilized RNA was hybridized to a mixture of LTR- and envelope-labeled DNA probes. An autoradiograph of the RNA·DNA hybrids from wild-type, resistant, and supersensitive T_1M_1 cell lines, without or with dexamethasone treatment is shown in Fig. 3. These experiments show that 35S, 24S, and 20S RNAs are present in these T_1M_1 cell lines. The 20S RNA hybridizes poorly to the envelope probe (data not shown). The 24S RNA/35S RNA ratio is very high, approximately 10, suggesting that either there is

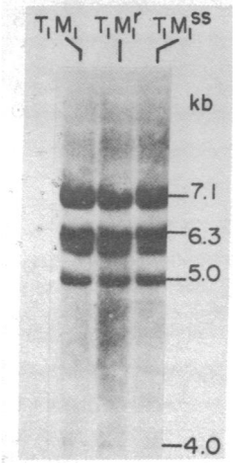

FIG. 2. Analysis of MMTV proviral DNAs from the T_1M_1 line and its $T_1M_1^r$ and $T_1M_1^{ss}$ variants. Cellular DNA from the various cell lines was cleaved with *Eco*RI and 10 μg of DNA was electrophoresed in 1% agarose gels. The DNA was then transferred from the gel to nitrocellulose and hybridized to the ^{32}P-labeled LTR fragment. Hybridized DNA fragments were visualized by autoradiography. Molecular weights indicated were determined by running *Pst* I-cut LTR, envelope, and pBR322 in a parallel lane.

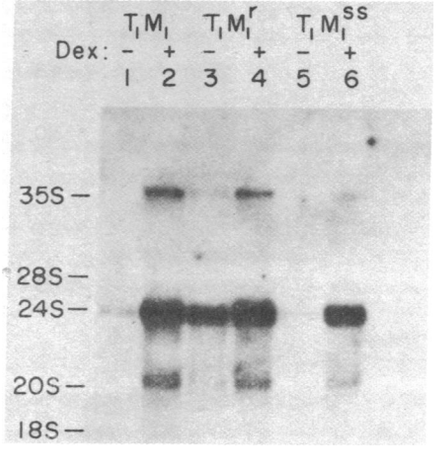

FIG. 3. Analysis of MMTV RNAs from T_1M_1 thymoma cell lines. Poly(A)$^+$RNAs (5 μg) from T_1M_1 (lanes 1 and 2), $T_1M_1^r$ (lanes 3 and 4), and $T_1M_1^{ss}$ (lanes 5 and 6) cells without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) dexamethasone treatment for 6 hr were electrophoresed in a 1.4% agarose gel. The RNAs were then transferred from the gel to nitrocellulose filters, hybridized with ^{32}P-labeled LTR and envelope cloned DNAs, and exposed for autoradiography for 5 days. The positions of the MMTV-specific RNAs are noted by their sedimentation constants, which were determined by electrophoresis of the rRNAs in a parallel lane (data not shown).

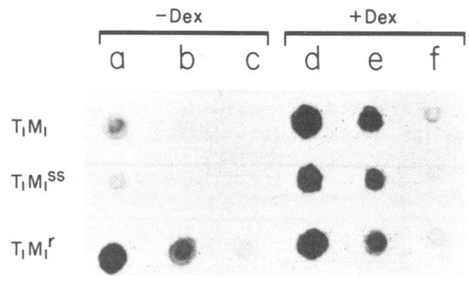

FIG. 4. MMTV RNA in dexamethasone (Dex)-sensitive, -supersensitive, and -resistant T_1M_1 cell lines. Dot blots were prepared with three amounts of poly(A)$^+$RNA: 5.2 μg (columns a and d), 1.3 μg (columns b and e), and 0.3 μg (columns c and f). RNAs were extracted from T_1M_1, $T_1M_1^{ss}$, and $T_1M_1^r$ cells untreated or treated with dexamethasone. Hybridization was carried out as described in Fig. 3 with the LTR and envelope DNA probes.

more envelope MMTV RNA synthesis than whole MMTV genomic RNA synthesis or there is a higher turnover of genomic RNA. The MMTV RNA content is increased after dexamethasone treatment in the wild-type and supersensitive T_1M_1 cell lines. In contrast, the MMTV RNA content of the resistant cell line is very high in the absence of hormone and is only slightly increased by dexamethasone treatment.

It is possible to quantify the total amount of MMTV RNA from these cell lines by liquid or "dot blot" hybridization (Fig. 4 and Table 1). In the absence of hormone, the amounts of MMTV are 50, 100, and 800 units, respectively, for the supersensitive, sensitive, and resistant cell lines. Short hormone treatment (6 hr) increases this amount by a factor of 10 in the supersensitive and sensitive cell lines but only slightly increases the MMTV RNA content of the resistant cell line. This is in agreement with measurements of glucocorticoid receptor in these lines: although the sensitive and supersensitive lines contain approximately 30,000 receptors per cell (4), the resistant cells contain only approximately 7,000 receptor sites.

Table 1. MMTV poly(A)$^+$RNA in supersensitive, sensitive, and resistant T_1M_1 thymoma lines

	$T_1M_1^{ss}$	T_1M_1	$T_1M_1^r$
Without dexamethasone	50	100	800
With dexamethasone	600	1,000	1,000

Poly(A)$^+$RNAs from untreated cell lines or cell lines treated for 6 hr with 0.1 μM dexamethasone were hybridized with either the LTR and envelope DNA probes or a cDNA probe. RNA was quantified by calculating the concentration of MMTV from C_rt curves. The 50% cDNA hybridization to pure viral RNA corresponds to $C_rt = 2 \times 10^{-2}$ mol·sec/liter. Results are expressed in molecules per cell and summarize several experiments.

Methylation of the LTR and Envelope MMTV Genes. Previous results with other systems suggest that gene expression is correlated with the unmethylated state of DNA as assayed by methylation-sensitive restriction enzymes (8). Thus, *Hpa* II is unable to cut C-C^m-G-G sites, whereas *Msp* I cuts these sites irrespective of cytosine methylation. Comparison of the different T_1M_1 variants showed no overall change in their DNA methylation as monitored by ethidium bromide staining of gels of *Hpa* II-digested DNA (data not shown).

The specific methylation at C-C-G-G sequences in MMTV proviral DNA can be determined by comparing the *Msp* I and

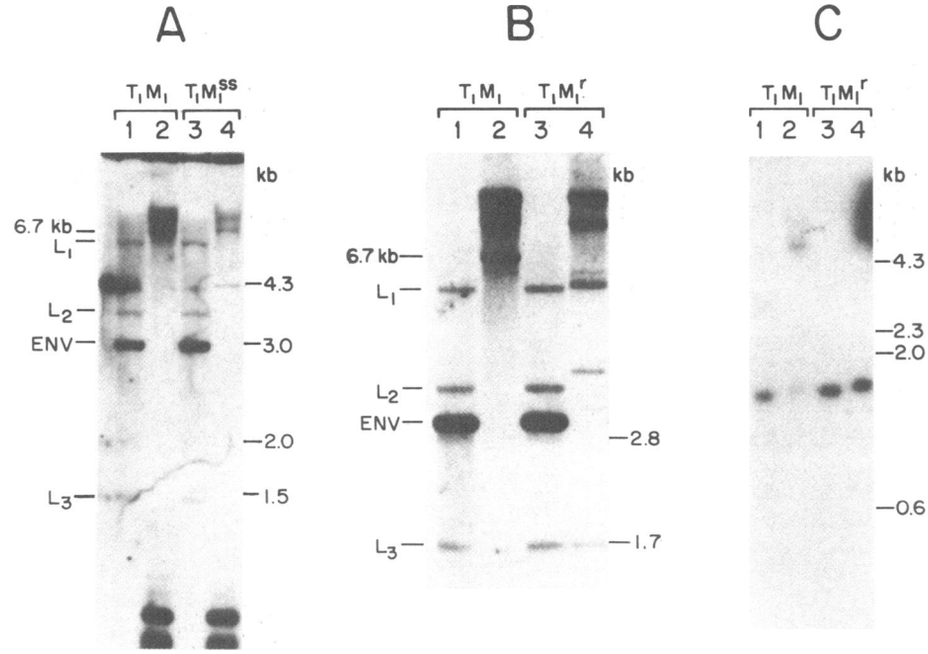

FIG. 5. Methylation of MMTV sequences in T_1M_1 cell lines. Cellular DNA from the designated cell lines was cleaved to completion with various restriction enzymes, and 10-μg samples were analyzed by Southern blot hybridization to LTR and envelope DNA probes as in Fig. 2. (*A*) DNAs extracted from T_1M_1 (lanes 1 and 2) and $T_1M_1^{ss}$ (lanes 3 and 4) were cut with *Msp* I (lanes 1 and 3) and *Hpa* II (lanes 2 and 4) and hybridized to the LTR and envelope probes. Completion of *Hpa* II digestion is shown by the low molecular weight bands generated by digestion of traces of pMB9 added to the DNA of lanes 2 and 4. Molecular weights (kb) of the *Pst* I fragments of the LTR and envelope (ENV) and of pBR322 are shown. (*B*) DNAs extracted from T_1M_1 (lanes 1 and 2) and $T_1M_1^r$ (lanes 3 and 4) were digested with *Msp* I (lanes 1 and 3) or *Hpa* II (lanes 2 and 4) and hybridized to the LTR and envelope probes. *Hpa* II activity was checked by inclusion of ϕX174 DNA in lanes 2 and 4, generating 2.8- and 1.7-kb bands that were visualized by staining. (*C*) DNAs extracted from T_1M_1 (lanes 1 and 2) and $T_1M_1^r$ (lanes 3 and 4) were digested with *Hha* I/*Msp* I (lanes 1 and 3) and *Hha* I/*Hpa* II (lanes 2 and 4) and hybridized to the LTR probe. L_1, L_2, and L_3, 5′-LTR fragments; ENV, the 3-kb fragment containing the 3′ LTR and the envelope gene (see Fig. 1).

Table 2. Methylation and size of DNA fragments containing LTR or envelope sequences

Cell line	L_1		L_2		L_3		ENV	
	Msp I	*Hpa* II	*Msp* I	*Hpa* II	*Msp* I	*Hpa* II	*Msp* I	*Hpa* II
T_1M_1 (wild type)	5.5	6.7	3.8	–	1.5	–	3.0	–
$T_1M_1^{ss}$ (supersensitive)	5.5	6.7	3.8	–	1.5	–	3.0	–
$T_1M_1^{r}$ (resistant)	5.5	5.8	3.8	4.5	1.5	1.5	3.0	–

L_1, L_2, and L_3, the 5′-LTR-containing DNA fragments identified in Fig. 5; ENV, DNA fragments containing the 3′ LTR and envelope sequences. Sizes are given in kb. –, No fragment was detected.

Hpa II digestion patterns of total DNA and the hybridization to proviral sequences. As shown in Fig. 1, MMTV proviral DNA contains one C-C-G-G site at the 3′ end of each LTR sequence and another such site in the envelope gene, giving a 3 kilobase (kb) DNA fragment (10). *Msp* I digestion will yield a 3-kb fragment containing the 3′ LTR and the envelope gene and other fragments containing the 5′-LTR and 5′-cellular sequences. The sizes of these 5′ fragments (L_1, L_2, and L_3) will vary with the location of the next *Msp* I site in the cellular genome. Therefore, *Msp* I digests will contain several 5′ fragments corresponding to each provirus integrated at a different site in the genome.

An autoradiograph of Southern blots of the *Msp* I and *Hpa* II-digested DNAs after hybridization with the LTR and envelope DNA probes is shown in Fig. 5. The results obtained with DNAs from wild-type T_1M_1 and $T_1M_1^{ss}$ are in Fig. 5*A*. The 3-kb envelope (ENV) fragment is present in all *Msp* I digests and absent in all *Hpa* II digests, indicating that one or both sites defining that fragment (Fig. 1) are methylated. *Msp* I digestion of the DNA of the T_1M_1 cell lines shows bands corresponding to the three 5′-end LTR DNA fragments (L_1, L_2, L_3). *Hpa* II digestion of the same DNAs shows only one band (6.7 kb), migrating faster than the high molecular weight DNA. This band hybridizes only with the LTR DNA probe and not with the envelope probe (results not shown). From these patterns, we conclude that only one 5′-LTR sequence is demethylated in the sensitive and the supersensitive T_1M_1 cell lines. The sizes of the 5′-end LTR DNA fragments are L_1, 5.5 kb; L_2, 3.8 kb; L_3, 1.5 kb. No difference is detected between the wild type and its supersensitive variant.

The MMTV DNA methylation of the T_1M_1 resistant cell line was analyzed by the same method. The methylation pattern of the LTR-containing fragments in the wild-type T_1M_1 line is compared with that of the resistant variant in Fig. 5*B*. Whereas the envelope gene is methylated, the LTR DNA fragments L_1, L_2, and L_3 are demethylated in the resistant variant because *Hpa* II digestion, which yields only one fragment in the parental line, yields three fragments in the resistant variant. The *Hpa* II fragment associated with L_1 is smaller in the resistant line than in the parent, suggesting demethylation of a site that was methylated in the parent. Both L_2 and L_3 fragments generate *Hpa* II fragments in the resistant line, indicating demethylation at or nearby the *Msp* I sites.

The results of double digestion with *Hha* I/*Msp* I and *Hha* I/*Hpa* II of DNAs from T_1M_1 and $T_1M_1^{r}$ hybridized with the LTR probe are shown in Fig. 5*C*. Because both *Msp* I and *Hha* I cut within the LTR (Fig. 1), this analysis specifically monitors the methylation of the *Msp* I site contained within the LTR. This site appears partially demethylated in the parent line (lanes 1 and 2) but fully demethylated in the resistant variant (lanes 3 and 4).

The results of MMTV DNA methylation and the size of the fragments obtained for the T_1M_1 lines are summarized in Table 2. The demethylated C-C-G-G sites of L_3 are very close to the extremities of the LTR sequence (1.5 kb). In contrast, the LTR L_1 and L_2 fragments of the resistant cell DNA contain *Hpa* II sites further upstream from the adjacent *Msp* I C-C-G-G site at 0.3 kb and 0.7 kb of this site, respectively.

To test a possible direct dexamethasone effect on the DNA methylation, we have treated resistant and sensitive cells with dexamethasone for 18 hr, equivalent to 1.5 cell doublings. Analysis of LTR and envelope DNA fragments showed no change in methylation (results not shown).

DISCUSSION

We have compared MMTV expression in several variants of the T_1M_1 cell line. T_1M_1 cells are killed by dexamethasone within 1 to 2 days. Two variants of the T_1M_1 line that have altered sensitivity to the cytolytic effect of dexamethasone have been examined: a supersensitive variant ($T_1M_1^{ss}$) that is killed faster by dexamethasone than the parental line (4) and a resistant variant ($T_1M_1^{r}$) that is deficient in glucocorticoid receptors and is not killed by glucocorticoids. MMTV expression is induced approximately 10-fold after 6 hr of treatment by dexamethasone in both the parental T_1M_1 line and the $T_1M_1^{ss}$ variant, while only a 20% induction was observed in the $T_1M_1^{r}$ variant (Table 1). This result is in agreement with the fact that the $T_1M_1^{ss}$ variant contains glucocorticoid receptor undistinguishable from that of the parental line (4) while the $T_1M_1^{r}$ variant has a smaller amount of glucocorticoid receptor. This result indicates that both the cytolytic response and MMTV induction are mediated by the glucocorticoid receptor. In hepatoma cells, induction of MMTV expression also requires a functional glucocorticoid receptor (18). The most striking result of the comparison between the parental T_1M_1 line and its variant is the very high constitutive level of MMTV expression observed in the resistant variant, $T_1M_1^{r}$, in the absence of glucocorticoids (Table 1). The possibilities that superinfection, site of provirus integration, or state of proviral DNA methylation could account for this elevated basal level of MMTV expression have been examined.

Analysis of *Eco*RI and *Msp* I digests of the $T_1M_1^{r}$ variant DNA indicate that the high constitutive expression of MMTV in this variant as compared with the parental line cannot be explained by superinfection or MMTV genome rearrangements, because the patterns of LTR and envelope fragments are identical in both lines. Comparison of *Hpa* II digests of the parental T_1M_1 line and the resistant $T_1M_1^{r}$ variant reveals that no overall genome demethylation can be detected by ethidium bromide staining and no specific demethylation was detected in the fragment containing the 3′ LTR and the envelope gene. However, demethylation did take place in the genome of the resistant variant at all three of the *Msp* I sites in the 5′ LTRs of the three proviruses (Fig. 5 *B* and *C*). This resistant variant was selected by continuous growth over a period of several weeks in the presence of a high concentration (10 μM) of dexamethasone. While we did not observe MMTV provirus demethylation after short (18-hr) dexamethasone treatment of the parental T_1M_1 line, our observations suggest that demethylation can occur by a mechanism involving several cell divisions in the presence of glucocorticoid. The continuous transcription of

MMTV in the T_1M_1 line that took place during selection of the variant might have played a role in these demethylation events. This interpretation is in agreement with a recent observation that estrogen stimulation results in demethylation at the 5′-end region of the chicken vitellogenin gene (19).

Numerous reports indicate a correlation between gene undermethylation and expression (8, 9, 20), although there are some exceptions (21, 22). In most cases, it is not known whether undermethylation is the cause or the consequence of gene expression. However, 5-azacytidine-induced demethylation can cause the expression of some genes (20, 23). In contrast, our results, as well as those of Wilks *et al.* (19), indicate that demethylation can be the consequence of prolonged hormone stimulation. It appears, therefore, that DNA undermethylation can both precede and follow gene expression. Whether the constitutive expression observed in the resistant variant is related to the demethylation events is unclear, but it is striking that demethylation occurred specifically at the same site—i.e., in the 5′ LTR—of the proviruses. Specific and permanent demethylation following long-term exposure to a steroid could conceivably play a role in hormone-induced differentiation.

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