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Dependence of Transcriptional Repression on CpG Methylation Density

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CpG methylation is known to suppress transcription. This repression is generally thought to be related to alterations of chromatin structure that are specified by the methylation. The nature of these chromatin alterations is unknown. Moreover, it has not been clear if the methylation repression occurs in an all-or-none fashion at some critical methylation density, or if intermediate densities of methylation can give intermediate levels of repression. Here I report a stable episomal system which recapitulates many dynamic features of methylation observed in the genome. I have determined the extent of transcriptional repression as a function of four densities of CpG methylation. I find that the repression is a graded but exponential function of the CpG methylation density such that low levels of methylation yield a 67 to 90% inhibition of gene expression. Higher levels of methylation extinguished gene expression completely. Transcription from methylated minichromosomes can be increased by butyrate treatment, suggesting that histone acetylation can reverse some of the repression specified by the methylated state. Sites of preferential demethylation occurred and may have resulted from transcription factor binding or DNA looping.

CpG methylation is important in mammalian development, as shown by recent work on methyltransferase knockout mice (19). CpG methylation is also an important regulator of gene expression in eukaryotic cells (reviewed in reference 3). Numerous studies have correlated the repression of transcription with CpG methylation (for reviews, see references 1 and 20). Studies based on in vitro assays, transient transfections, or stable integrations have attempted to dissect the relationship between methylation, chromatin structure, and gene expression (2, 4, 5, 14, 17). The correlation is thought to be based on chromatin effects that either precede or follow the CpG methylation; however, the specific state of the chromatin and the differences in that chromatin in methylated regions of DNA are uncertain. Many global aspects of the association between methylation and gene expression remain to be defined. In particular, it is unclear whether the effect of CpG methylation is graded versus all or none. That is, does the methylated state correlate with transcriptional repression in a linear or a highly exponential fashion?

Obtaining an understanding of eukaryotic CpG methylation has been difficult because of the lack of an easily manipulated genetic system. It has been suggested that the interaction of CpG methylation, DNA replication, and chromatin structure may be important for defining a silent state of genes (3, 13). In an effort to understand the role of CpG methylation in DNA replication, transcription, and chromatin structure, an episomal system based on Epstein-Barr virus (EBV) was used. It has been well established that plasmids bearing the EBV replication origin (oriP) can be stably maintained in human cells transformed with the virus or human cells containing the gene for EBV nuclear antigen EBNA1 (15, 21, 25, 26). A stable episomal system using the EBV replication machinery can be easily manipulated and analyzed. Therefore, long-term in vivo observation is possible.

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In this study, the level of gene expression as a function of the degree of CpG methylation was determined. Minichromosomes with four different CpG densities, i.e., 0% methylation, 7% methylation (CpG sites at all *Hha*I sites), 23% methylation (CpG sites at all *Hha*I, *Hpa*II, and *Fnu*DII sites), and 100% methylation, were examined. In addition, chemical modifiers of the repressed state were studied. 5-Azacytidine (5-azaC) has been shown to cause some degree of hypomethylation in many mammalian systems (10, 16). Sodium butyrate inhibits histone deacetylation, and this leads to the relaxation of nucleosomal DNA and induction of transcription (22). Finally, the stability of unmethylated and methylated minichromosomes and the maintenance of CpG methylation as a function of time in eukaryotic cells were examined. These studies are important because they provide a quantitative determination of the relationship between the extent of CpG methylation and gene expression.

MATERIALS AND METHODS

Plasmid. Plasmid pCLH22 (Fig. 1) was constructed by inserting the luciferase gene with the Rous sarcoma virus long terminal repeat (RSV LTR) promoter, a blunt-end *Nde*I-*Bam*HI fragment from pRSVL (6), into the *Bam*HI and *Hin*dIII sites of p220.2 (7).

In vitro DNA methylation. DNA was methylated with *Hha*I or CpG methylase for more than 3 h under conditions recommended by the manufacturer (New England Biolabs). DNA was phenol-chloroform extracted and ethanol precipitated after in vitro methylation. The efficiency of methylation was confirmed by enzyme digestion and by transformation into a strain of methylation-restrictive bacteria, *Escherichia coli* DH5α. The triple methylation was done by methylating the plasmid sequentially with *Hpa*II, *Hha*I, and *Fnu*DII methylases, with phenol-chloroform extraction and ethanol precipitation after each methylation step. The methylation sites are illustrated in Fig. 2. There are, on average, one methylated CpG site every 274 bases within the luciferase gene, one every 150 bases within the hygromycin gene, one every 173 bases in

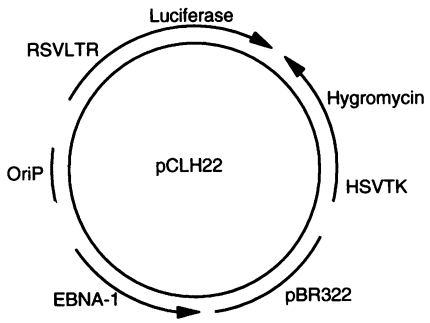

FIG. 1. EBV-based plasmid. This plasmid carries the prokaryotic replication origin and the β-lactamase gene from pBR322. Arrows indicate the direction of transcription for the various elements. HSVTK, promoter for the hygromycin gene; RSVLTR, promoter for the luciferase gene; OriP, EBV-based eukaryotic replication origin.

the prokaryotic sequences, and one every 798 bases within the EBV sequences on the *Hha*I-methylated minichromosome. The *Hha*I density in the luciferase gene region is the most representative of the average *Hha*I density of the entire minichromosome (one every 300 bases).

Cell line. A derivative of the 293 human embryonic kidney carcinoma cell line was used in this study. This 293/EBNA1 cell line was derived by integrating EBNA1 sequences into 293 cells. The cells were grown in Dulbecco modified Eagle medium with 10% fetal calf serum and penicillin-streptomycin.

Transfection. The calcium phosphate transfection method (24) was used throughout this study. A concentration of 2 μg of DNA per ml of transfection mix, which contains *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer and 124 mM calcium chloride, was used. The cells were seeded in 35-mm-diameter tissue culture plates 2 days prior to the transfection. For each 35-mm-diameter plate at a cell density of 30 to 50% confluency, 170 μl of transfection mix was used. All experiments were done in duplicate.

The cells were incubated with transfection mix for 16 h. Fresh medium was added to the transfected cells after two washes with phosphate-buffered saline.

Harvest and analysis. When the transfected cells reached confluence, 10% of the cells were harvested for luciferase analysis, 40% of the cells were plated in a 100-mm-diameter tissue culture plate without selection, and 50% of the cells were replated in the 35-mm-diameter plate with hygromycin selection (final concentration, 400 μg/ml). The hygromycin-treated plates were for the assessment of transfection efficiency and not for further experiments. When the cells reached confluence in the 100-mm-diameter plate, 1.25% of the cells were harvested for luciferase analysis, 5% of the cells were replated in a 100-mm-diameter plate, and the rest of the cells were harvested for plasmid DNA.

The plasmid DNA was harvested from the transfected 293/EBNA1 cells by the Hirt method (11). One-fourth of the DNA from each harvest was digested with *Xba*I to linearize the plasmid, and an equal amount of the DNA was doubly digested with *Xba*I and *Hha*I to determine the methylation status. The digested DNA was fractionated on 0.8% agarose gel, Southern transferred onto a nylon membrane, and probed with the entire plasmid. To identify loss of methylation at specific sites on the plasmid, specific fragments were isolated and used to reprobe the blots. The autoradiogram was quantitated with a model 300A computing densitometer (Molecular Dynamics).

Luciferase expression analysis. An aliquot of the transfected cells was harvested and lysed for luciferase activity analysis. The luciferase activity was analyzed on a luminometer as described previously (12). In this study, different transfected cell populations take up different amounts of plasmid DNA. Therefore, the measurement of luciferase gene expression must be normalized by the amount of plasmid DNA in each of the transfected cell populations. That is, the gene expression was normalized for the same quantity of plasmid DNA with different methylation states.

5-AzaC and sodium butyrate treatment. 5-AzaC was added to the transfected cells at a final concentration of 8 or 16 μM

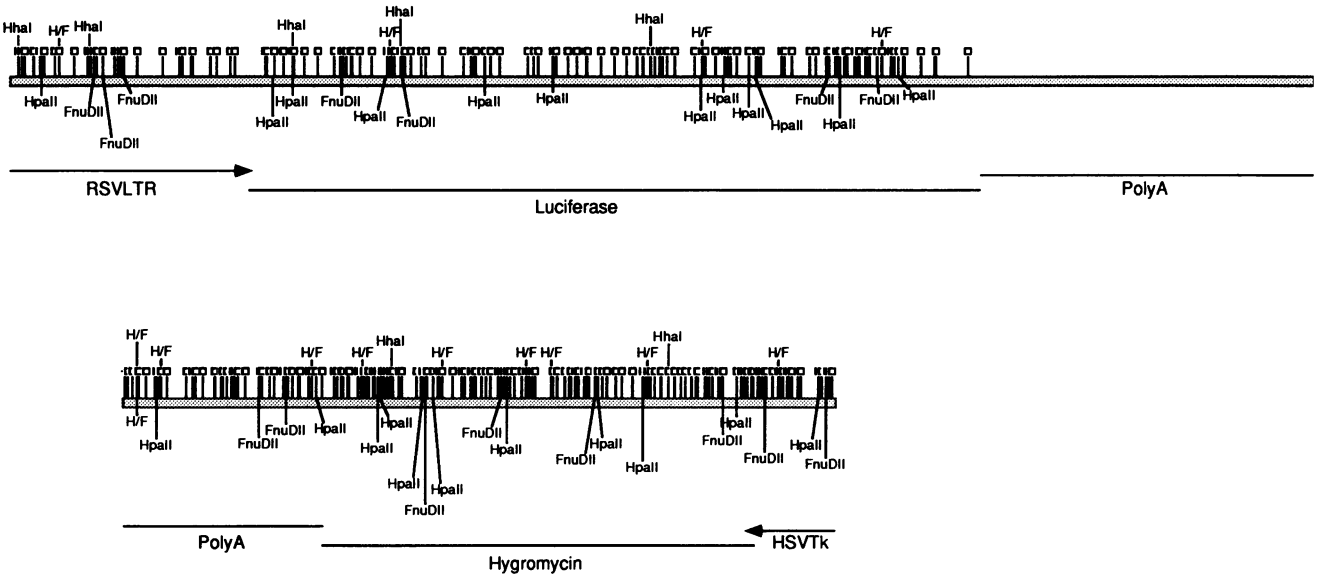

FIG. 2. CpG, *Hha*I, *Hpa*II, and *Fnu*DII sites in the region of the luciferase gene and hygromycin gene on plasmid pCLH22. The CpG sites are marked with squares, the overlapping *Hha*I and *Fnu*DII sites are marked with H/F, and *Hha*I, *Hpa*II, and *Fnu*DII sites are as indicated.

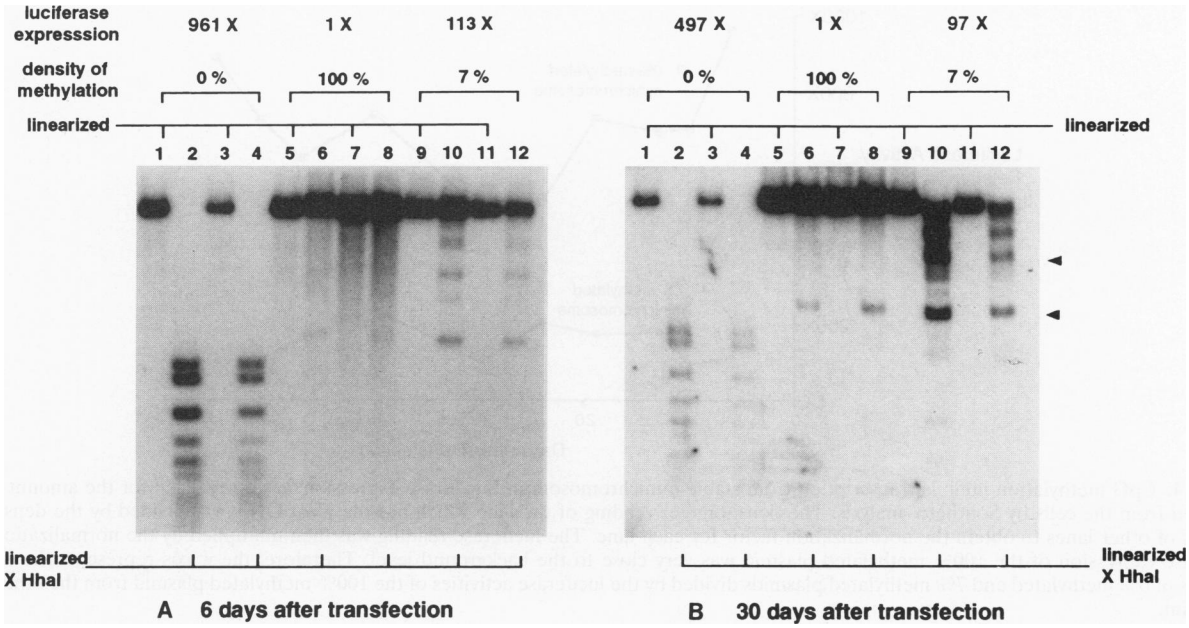

FIG. 3. Long-term maintenance of CpG methylation and preferential demethylation. Odd-numbered lanes are linearized (by *Xba*I digestion) plasmid DNA from transfected cells, and even-numbered lanes are doubly digested (by *Xba*I and *Hha*I) plasmid DNA from transfected cells. (A) Plasmid DNA harvested 6 days after transfection; (B) plasmid DNA harvested 30 days after transfection. Lanes: 1 to 4, 0% methylated pCLH22; 5 to 8, 100% methylated pCLH22; 9 to 12, 7% methylated pCLH22. Arrowheads mark the products from preferential demethylation.

for various lengths of time. Sodium butyrate was added to the transfected cells at a final concentration of 10, 20, or 25 mM at 16 h before harvest.

RESULTS

Stability of CpG methylation on minichromosomes. Three densities of CpG methylation (0, 7, and 100%) were examined for the efficiency with which the CpG methylation is maintained on stable episomes in human fibroblasts. Unmethylated (0% methylated), completely CpG methylase-methylated (100% methylated), and *Hha*I methylase-methylated (7% methylated) plasmids were transfected into human fibroblasts (293/EBNA1 cells). The methylation status of the plasmids was determined by *Hha*I-*Xba*I double digestion and compared with *Xba*I digestion of the harvested DNA. The episomes were studied throughout a 60-day experimental time course. The 0% methylated minichromosomes did not gain CpG methylation at any *Hha*I sites throughout the entire 60-day period (Fig. 3). The 100% methylated minichromosomes and 7% methylated minichromosomes retained methylation at almost all *Hha*I sites for the 60-day period of analysis (Fig. 3). The 100% methylated minichromosomes also maintained methylation at almost all of the *Hpa*II sites (data not shown) throughout the study. Although these assessments do not examine every CpG site on the 100% methylated minichromosomes, it is most likely that methylation of the large majority of the CpG sites on the minichromosomes is maintained by the endogenous methyltransferase. Furthermore, the unmethylated minichromosomes do not become CpG methylated.

Nuclear retention of CpG-methylated minichromosomes. It was of interest to determine how well minichromosomes with and without methylation are retained in the cell nuclei. Methylated minichromosomes cannot be maintained by hygromycin selection in mammalian cells because the methylation inhibits their transcription. Hence, this experiment, like all experiments in this study, was conducted without selection. Interestingly, completely methylated minichromosomes were more stable than 0 and 7% methylated minichromosomes in the human fibroblasts. Approximately equal amounts of 0% methylated and 7% methylated plasmid DNA were harvested at 6 days after transfection (less than a twofold difference). Five- to 10-fold more and 15-fold more 7% methylated than 0% methylated DNA were harvested at 30 days (Fig. 3) and 55 days (data not shown) after transfection, respectively. The same trend held for the 100% methylated versus 0% methylated minichromosomal DNA. Hence, minichromosomes with partial or complete CpG methylation were considerably more stable in the cells than unmethylated minichromosomes.

Transcription is dramatically inhibited by 7% or more CpG methylation. In previous studies of the relationship between transcription and CpG methylation, it has never been clear whether the inhibitory effect of methylation on transcription is exponential or linear. To address this issue, I analyzed luciferase gene expression at 0, 7, 23, and 100% levels of CpG methylation. In this study, the measurement of gene expression was normalized for the amount of plasmid DNA in the transfected cells so that expression from the same quantity of DNA could be compared. After being normalized for the amount of plasmid DNA in the cells, luciferase gene expression from the 0% methylated minichromosomes was 3- to 10-fold higher than that from the 7% methylated minichromosomes and 500- to 1,000-fold higher than that from the 100% methylated minichromosomes throughout the experiment (Fig. 4). This finding indicates that gene expression is 67 to 90% reduced by methylating 40 CpGs at *Hha*I sites on the plasmid (7% methylated minichromosome) and is eliminated by complete CpG methylation (Fig. 5). When the plasmid is triply methylated by *Hpa*II, *Hha*I, and *Fnu*DII methylases sequentially, 23% methylation (130 sites) can be achieved. The gene expression from the 23% methylated pCLH22 was 90- to

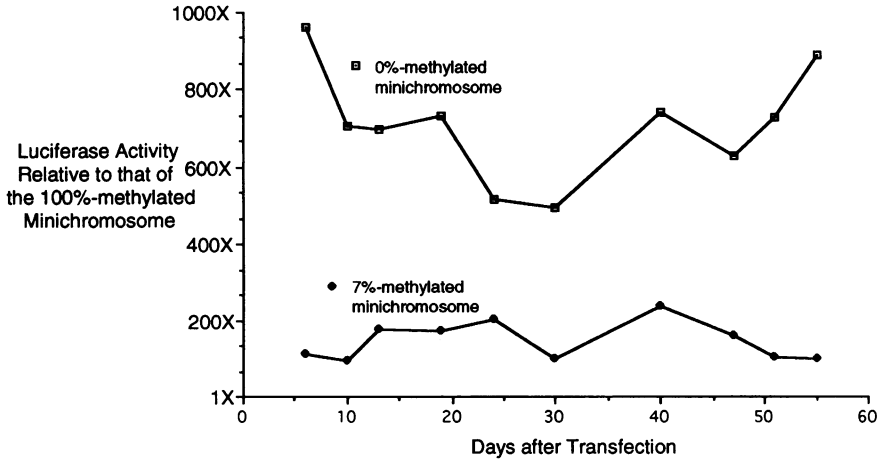

FIG. 4. CpG methylation inhibits transcription from stable minichromosomes. Luciferase expression was normalized for the amount of DNA harvested from the cells by Southern analysis. The densitometer reading of the lane which has the most DNA was divided by the densitometer readings of other lanes to obtain the normalization factor for each lane. The luciferase reading was then multiplied by the normalization factor. Luciferase expression of the 100% methylated plasmid was very close to the background level. Therefore, the *y* axis represents the luciferase activities of 0% methylated and 7% methylated plasmids divided by the luciferase activities of the 100% methylated plasmid from the same harvest time point.

190-fold lower than that from 0% methylated pCLH22. This can be translated into a 99 to 99.5% reduction of transcription by the 23% CpG methylation (Fig. 5). I conclude that 7% or more CpG methylation can dramatically inhibit transcription and that 100% CpG methylation can abolish any measurable level of gene expression.

Transcriptional induction is progressive and irreversible upon treatment of methylated minichromosomes with 5-azaC. To determine how the demethylating agent 5-azaC would affect transcription from minichromosomes with different degrees of methylation, transfected cells were treated with 5-azaC at a final concentration of 16 μM for 16 h, and gene expression was assessed. The luciferase activity was unchanged in the treated and untreated cells for the 0% methylated and 7% methylated plasmids after treatment. For the 100% methylated minichromosomes, gene expression was increased two- to threefold above the baseline value (Fig. 6). Therefore, 5-azaC treatment had little effect during the first 16 h of treatment.

To examine the long-term effect of 5-azaC on the dynamics of any demethylation and transcription, a portion of the cell population treated with 5-azaC for 16 h in the preceding experiments was divided into two groups. One group was subjected to continuous treatment with 5-azaC at a final

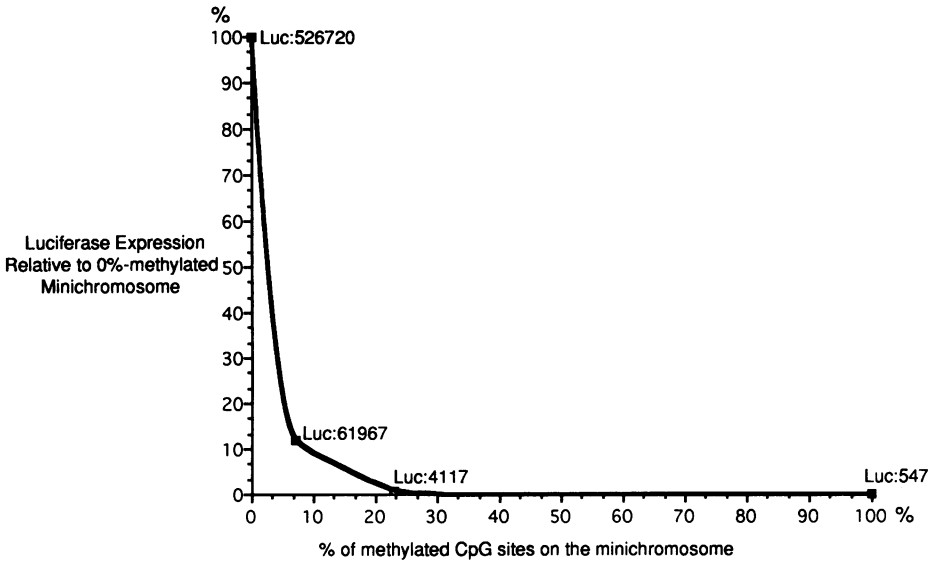

FIG. 5. Transcription inhibition and extent of methylation. The *y* axis represents the transcription level of the minichromosomes 6 days after transfection, using the 0% methylated minichromosome luciferase value to represent 100% gene expression. The luciferase expression of each form of minichromosomes was normalized for quantity of DNA in the transfected cells. The *x* axis represents the proportion of methylated CpG sites on the minichromosomes.

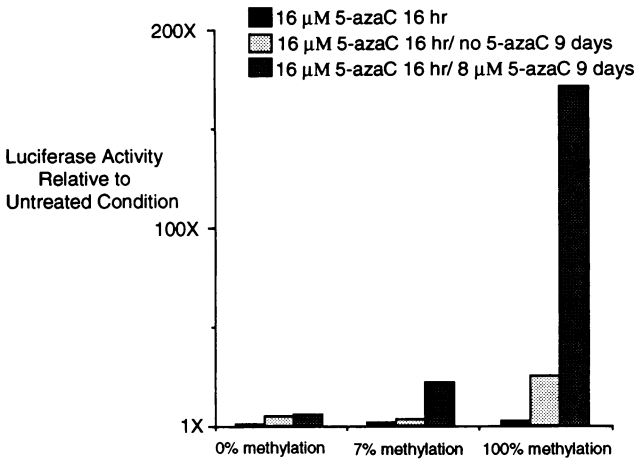

FIG. 6. 5-AzaC induces transcription from stable minichromosomes. The relative luciferase expression in 5-azaC-treated cells was derived by dividing the luciferase reading of treated cells by that of untreated cells from the same transfection.

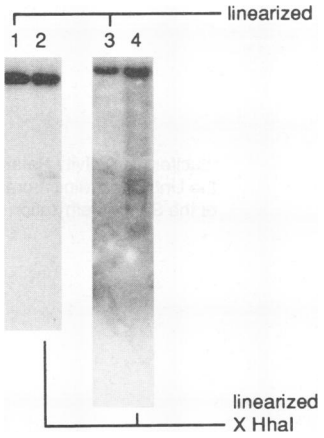

FIG. 7. Demethylation at some of the *Hha*I sites on the 100% methylated minichromosomes. Lanes: 1 and 3, linearized (by *Xba*I digestion) plasmid DNA from transfected cells; 2 and 4, doubly digested (by *Xba*I and *Hha*I) plasmid DNA from transfected cells; 1 and 2, plasmid DNA harvested from cells without 5-azaC treatment; 3 and 4, plasmid DNA from cells treated with 16 μM 5-azaC for 16 h and then with 8 μM 5-azaC for 9 days before harvesting.

concentration of 8 μM for 9 days, while control cells in the other plate were not further treated with 5-azaC. The cells not treated with 5-azaC after the initial 16 h of 5-azaC treatment showed a 4- to 5-fold increase in gene expression for 0% methylated minichromosomes, a 3- to 4-fold increase for 7% methylated minichromosomes, and a 20- to 25-fold increase for 100% methylated minichromosomes compared with untreated cells carrying the corresponding minichromosomes (Fig. 6). The 3- to 5-fold effects seen for the 0% methylated and 7% methylated minichromosomes presumably reflect pleiotropic, delayed effects of 5-azaC on the cells. The larger impact on the 100% methylated minichromosomes indicates an effect specific to the highly methylated state. The effect of the initial 16 h of 5-azaC treatment persists and, in fact, increases over the subsequent 9 days despite the absence of 5-azaC.

The cells with 9 more days of 5-azaC treatment showed a 5- to 6-fold increase for 0% methylated, a 15- to 28-fold increase for 7% methylated, and a 130- to 200-fold increase for 100% methylated minichromosomes compared with untreated cells (Fig. 6). Hence, prolonged 5-azaC treatment has an even greater impact on transcription.

Southern analysis after 5-azaC treatment showed that a portion of the 7 and 100% methylated plasmids were demethylated at some of the *Hha*I sites (Fig. 7). This result implies that the increase of transcription was most likely the consequence of partial or complete demethylation of some of the minichromosomes. This conclusion is also supported by the fact that the level of transcription from 7% methylated minichromosomes never reached that of the 0% methylated minichromosomes.

Butyrate increases transcription from methylated and unmethylated minichromosomes in a reversible fashion. Sodium butyrate is known to cause histone acetylation, which increases the mobility of nucleosomes on the DNA and increases transcription (18, 22). To determine whether histone acetylation could increase transcription from minichromosomes in the methylated state, sodium butyrate was added to the cells 16 h prior to harvest at a final concentration of 10, 20, or 25 mM. Compared with untreated cells, the luciferase activity increased 16- to 21-fold for 0% methylated minichromosomes, 50- to 65-fold for 7% methylated minichromosomes, and 15- to 34-fold for 100% methylated minichromosomes (Fig. 8). The level of transcription from the 7% methylated minichromosomes reached that of the 0% methylated minichromosomes upon sodium butyrate treatment.

To examine the reversibility of these effects, a portion of the treated cell population was replated and cultured without sodium butyrate for 5 days. The luciferase activity decreased to near its original, nontreatment level (data not shown). These findings indicate that sodium butyrate can induce transcription, and the effect of sodium butyrate on transcription is reversible within 5 days. No demethylation at the *Hha*I sites was observed by Southern blot analysis of the treated DNA. Therefore, the increase in transcription can occur without any measurable effect on methylation (see below).

Preferential demethylation at specific CpG sites. Over the 60-day period of analysis, almost all of the *Hha*I sites that were initially methylated remained methylated by Southern blot analysis. Hence, the methylated state of the EBV episome is very stable. Nevertheless, there were two small regions of the minichromosome which lost their methylation. These demethylation sites were determined by using specific *Hha*I-digested fragments of the plasmid to reprobe the Southern blots. One of these demethylation sites occurred in a small subpopulation of the 7 and 100% methylated minichromosomes. The other occurred in a larger subpopulation of the 7% methylated minichromosomes. In both cases, the fraction of the subpopulation of demethylated plasmids increased over time (Fig. 3). The site of preferential demethylation in the 100% methylated minichromosomes is at one to three closely spaced *Hha*I sites at nucleotide positions 10739, 10747, and 10787 of the 12,158 bp in the minichromosome. These are the only three *Hha*I sites in oriP of the minichromosomes and are adjacent to the region containing a multimer of 30-bp repeats (family of repeats) where EBNA1 binds. The resolution of the gel used did not allow determination of which of these sites was demethylated. In addition to this region, a *Hha*I site in the herpes simplex virus (HSV) *tk* promoter region (base 5219) was also preferentially demethylated in the 7% methylated minichromosomes. These results indicate that some sites can become demethylated even though all of the surrounding sites remain methylated.

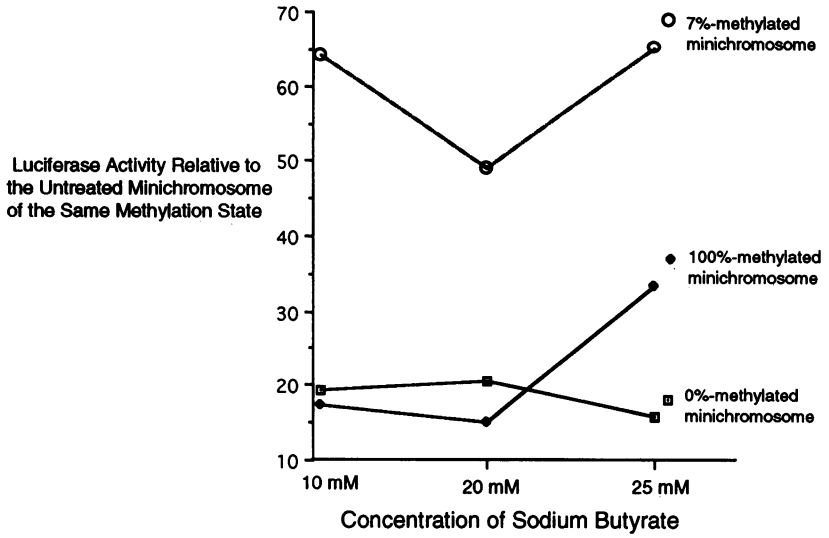

FIG. 8. Butyrate induces transcription from stable minichromosomes. The relative luciferase expression in butyrate-treated cells was derived by dividing the luciferase reading of treated cells by that of untreated cells from the same transfection.

DISCUSSION

The following features of CpG methylation are observed in these studies. First, transcriptional repression is dramatic even when only 7% of the CpG sites are methylated. Second, the 0, 7, and 100% levels of CpG methylation are stably transmitted over 60 days. Methylation is not readily lost on the replicating 7% methylated (with low but significant transcription) and 100% methylated (without detectable transcription) minichromosomes. Methylation is not gained on 0% methylated DNA that is replicating and transcribing. Third, the nuclear retention of 7% and 100% methylated minichromosomes is much higher than for 0% methylated minichromosomes. Fourth, in the presence of prolonged 5-azaC treatment at a concentration of 8 μM, some demethylation occurs and transcription increases. This effect persists well after the withdrawal of 5-azaC. Fifth, sodium butyrate induction of transcription can occur in spite of the methylation, and this effect is reversible after butyrate withdrawal. Sixth, some *Hha*I sites show preferential demethylation despite the persistence of methylation at the surrounding *Hha*I sites.

The modulation of transcription on these minichromosomes is interesting. The fully methylated state is not active for transcription, consistent with previous studies demonstrating this point (4, 5). Methylation binding proteins have been suggested to be the basis for this finding. More interestingly, I find that transcriptional activity decreases dramatically with the methylation of only a limited number of CpG sites. How transcription is modulated by only 7% CpG methylation is uncertain, and a stable, multicopy minichromosome model will be important in permitting analytical experiments to define the biochemical constituents of the chromatin that compose this repressed state. This episomal system can be used to analyze many aspects of CpG methylation because of the stable maintenance of CpG methylation on the minichromosomes in mammalian cells demonstrated in this study.

In this study, I have tried to define the dynamic range of this system. The 5-azaC treatment in this system resulted in some detectable demethylation but not complete demethylation of the minichromosomes. Though 5-azaC has been thought to act by interfering with methyltransferase activity, dramatic changes in methylation status of genetic loci are not found in the majority of cases. Studies that have found a detectable demethylation of endogenous loci have found it to be very limited in extent. Nevertheless, 5-azaC treatment in this study and in other studies does increase transcription. The minichromosome system described here permits the methylated, partially methylated, and unmethylated states to be compared in the same cell line in a way that has not been possible previously. I find that despite the absence of significant demethylation, transcription increases 130- to 200-fold for the 100% methylated state upon treatment with 5-azaC. It should be noted that this higher level of transcription is still 5- to 20-fold below the transcription level of the 0% methylated minichromosomes. On the basis of the Southern blot analysis, I propose that a subpopulation (perhaps 5 to 20% of the population) of minichromosomes is demethylating to an extent sufficient to account for the expression found for the 5-azaC-treated, methylated minichromosomes.

I find that the partially repressed state of 7% methylated minichromosomes can be activated in a reversible fashion by addition of sodium butyrate. Butyrate treatment can have pleiotropic effects, but its major site of action relevant to transcription is thought to be acetylation of the histone tails of H2A and H2B. This acetylation in the nucleosome octamer loosens the electrostatic interaction between the octamer and the phosphate backbone, permitting transcription factors necessary for initiation to bind at their recognition sites (18). When the butyrate is withdrawn, transcription returns to its lower, repressed state. These results indicate that the effect of butyrate can reverse part of the repression due to methylation in that transcription from 100% methylated minichromosome increases. The increases in transcription from 0% and 100% methylated minichromosomes are of the same magnitude, but transcription from 100% methylated minichromosomes was still more than 700-fold lower than that from the 0% methylated minichromosomes. Interestingly, the increase of transcription for the 7% methylated state is considerably larger upon butyrate treatment, and the level of transcription was comparable to that of 0% methylated minichromosomes. The basis for the difference between the 7% and 100% methylated

minichromosomes is not understood. One possible explanation is that 100% methylated minichromosomes acquired a more complex chromatin structure which cannot be altered to the same extent as that of the 7% methylated minichromosomes. The alteration of chromatin structure of 100% methylated minichromosomes allows only a very low level of transcription to occur. For the 7% methylated minichromosomes, the increase in transcription may have been due to a combination of upregulation of general overall cellular transcription and the alteration of chromatin structure specified by this limited (7%) methylation.

Both 5-azaC and butyrate have pleiotropic effects on cells. In the 5-azaC and butyrate studies, the possibility of *trans* effects such as upregulation of transcription or protein processing factors cannot be ruled out. However, luciferase expression from the 0% methylated minichromosomes increases only slightly with 5-azaC or butyrate treatment, which indicates that the impact of *trans* effects on gene expression is likely to be small for these treatments. Further studies using endogenous genes will elucidate the *trans* and *cis* effects on expression of methylated loci upon 5-azaC and butyrate treatment.

Though the methylation status is remarkably stable, there are specific sites that preferentially demethylate. It is intriguing that one of the preferred demethylation sites on both the 100 and 7% methylated minichromosomes is between the two EBNA1 binding sites. Could EBNA1 binding interfere with the maintenance methylation process? It has been shown that the dyad symmetry and the family of repeats in oriP form a complex with EBNA1, which results in a DNA loop that activates replication from oriP (reference 9 and references therein). This looping may have made the intervening DNA inaccessible to methyltransferase and resulted in the demethylation. The system here will allow such issues to be addressed.

The other preferential demethylation site on the 7% methylated minichromosomes is in the HSV *tk* promoter. Could this be the result of competition between transcription factors and methyltransferase? The fact that the HSV *tk* promoter site becomes demethylated in the 7% methylated minichromosomes, which are transcriptionally active, but not on the 100% methylated minichromosomes, which are not transcriptionally active, suggests that the effect may be due to transcription factor binding. Otherwise, there is no apparent reason why this same site should not be demethylated on the 100% methylated minichromosomes. The determination of demethylation at the HSV *tk* promoter site in the 7% methylated minichromosomes is clearly not sequence or DNA structure dependent; if it were, the 100% methylated minichromosomes should also demethylate at this site. Yet the processes of transcriptional initiation and elongation are also clearly not sufficient to target a region for demethylation. If this were the case, the 7% methylated minichromosomes, which are active for gene expression, should become efficiently demethylated over a considerable region surrounding the hygromycin gene as well as luciferase gene and the RSV LTR promoter controlling it. They are not. Moreover, butyrate induction, which increases the gene expression 15- to 34-fold from the 7% and 100% methylated minichromosomes, should cause demethylation of the luciferase and hygromycin genes and their promoters. It does not. Therefore, transcriptional initiation and elongation per se do not seem to target a region for demethylation. The specific demethylation at the HSV *tk* promoter on the 7% methylated minichromosomes may be due to competition between a protein which binds at or near that specific *Hha*I site and the maintenance methylase. The failure of this site to demethylate on the 100% methylated minichromosomes may be due to the complete inaccessibility of the 100% methylated minichromosomes to any such binding protein.

A final point is that the nuclear stability (retention) of the methylated minichromosomes is much greater than that of the unmethylated minichromosomes. Even the partially methylated minichromosomes were maintained substantially more efficiently than the unmethylated minichromosomes. This is the first demonstration of an association between CpG methylation and minichromosome stability. One can only speculate on the functional significance of this observation. One possibility is that partial or complete methylation contributes to a degree of chromatin structure that is more recognizable by the mechanisms that retain DNA in the nucleus. This might be important for making the retention of foreign DNA, which does not have CpG methylation, less efficient. The other possibility is that methylation is important for proper segregation of the genetic material in the cell. A segregation distortion phenotype associated with 70% reduced 5-methylcytosine in *Arabidopsis thaliana* and aneuploidy in methylation-deficient *Neurospora* mutants have been reported (8, 23). Both of these studies indicate that methylation may be a critical component involving chromosome segregation.

I recognize that these minichromosomes are composed of DNA segments that are not normally contiguous or even present in any single eukaryotic genome. Moreover, this artificial system may not reflect all of the effects of DNA methylation on endogenous genes. I could have taken a specific endogenous mammalian gene and its surrounding DNA and placed it on an EBV episome and monitored its time course of expression and demethylation. I am currently doing this. However, the dynamics of CpG methylation are relevant to the entire vertebrate genome; hence, they are not highly sequence dependent. For that reason, I felt that the dynamics that were monitored in this study and the inferences that I have drawn from these dynamics are of general relevance. The fact that an average of one methylated CpG site per 300 bases can markedly repress transcription raises the possibility that this level of methylation is adequate to keep genes silent when they are not being utilized.

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