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Stability of Patch Methylation and Its Impact in Regions of Transcriptional Initiation and Elongation

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CpG DNA methylation has previously been correlated with the suppression of transcription. The mechanism of this suppression is not understood, and many aspects of the temporal and positional relationships between the region of methylation and transcription have not yet been defined. Here, 12-kb stable replicating episomes that can be maintained in human somatic cells for weeks to months were used. Such a system allows more direct manipulation and is free from the positional effects attendant with the analysis of endogenous loci or integrated transgenes. By using these circular minichromosomes, patches of CpG methylation were created to include or exclude the regions of transcriptional initiation and elongation. I found that a 0.5-kb patch of methylation that covered the promoter suppressed expression only 2-fold and that a 1.9-kb patch of methylation that covered the coding portion of the gene (but not the promoter) suppressed expression about 10-fold. In contrast, methylation of the entire minichromosome except for the promoter or the coding portion suppressed transcription about 50- to 200-fold. I infer the following. Methylation of the 0.5-kb promoter fragment does not significantly affect transcription at the level of transcription factor binding or local chromatin structure. The dominant effect on transcription occurs when the length of methylated DNA is long, with little disproportionate effect of methylation of specific regions, such as that of initiation or elongation. I also found that the boundaries between these methylated and unmethylated regions remained stable for the many weeks that I monitored them.

CpG methylation has previously been associated with reduced transcription (see references 2 and 23 for reviews), decreased DNase I sensitivity (16), and decreased site-specific recombination (10). The repressive aspects of chromatin structure specified by CpG methylation have not yet been identified.

CpG methylation is tightly regulated during replication and differentiation of somatic cells. A maintenance methyltransferase functions during DNA replication to preserve the methylated pattern of preexisting methylated regions (18) (for a review, see reference 3). In general, genes lose CpG methylation within the promoter and in the transcribed region of the gene when they become activated, whereas genes acquire CpG methylation when they are no longer transcribed (for reviews, see references 4 and 24). However, it is not clear how regions of DNA become demethylated, how they acquire methylation in somatic cells, and over what time intervals these changes occur.

Many basic questions regarding CpG methylation remain unanswered. Does methylation specifically in the regions of initiation or elongation inhibit the transcription process? Do methylation of the promoter and methylation of the coding region of the gene have different effects on transcription? What length and density of CpG methylation are sufficient to affect the transcriptional activity of an adjacent unmethylated region? Is the boundary between a methylated region and an unmethylated region stable when there are no changes in transcriptional activity? What is the stability of a methylation boundary over time? Specifically, does CpG methylation spread into an adjacent unmethylated region? Reciprocally, does an unmethylated region erode methylation in an immediately adjacent methylated region over time? Although some of these questions have been studied previously (7, 15, 17, 22), transient assays, in vitro transcription, and substrate integration were used. It has previously been shown that chromatin assembly is typically coupled with DNA replication (13, 25) and that replication-coupled chromatin assembly is required for basal transcription suppression (1). An appropriate chromatin structure is lacking in nonreplicating transient and nonreplicating in vitro assays. In approaches that use chromosomal sites, positional effects cannot be ruled out at different locations. Therefore, the specific level of methylation inhibition of transcription cannot be reliably addressed by these approaches.

I have developed a stable episomal system to study the dynamics of CpG methylation over several months in mammalian cells (9). The stable episome utilizes the replication origin of Epstein-Barr virus (EBV), *oriP*. Each minichromosome maintains the CpG methylation pattern that it possessed at the time of transfection. This indicates that the maintenance methyltransferase efficiently remethylates the newly synthesized strand at positions opposite the existing sites of CpG methylation. Each minichromosome functions as an independent unit that is free from the positional effects attendant with chromosomally integrated reporter constructs or transgenes. Using this system, I have previously described the dependence of transcriptional activity on CpG methylation density and demonstrated the resemblance of this minichromosome to the endogenous genes in 5-azacytidine and sodium butyrate responses. Many questions related to the dynamics of CpG methylation have been difficult to address due to the complexity of the genome and the lack of a reliable genetic system. This is the only stable episomal system to date that allows us to answer some of these fundamental questions and to dissect temporal versus causal effects of DNA methylation on various processes in mammalian cells.

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In this study, I used the previously described stable replicating minichromosomes to examine the following questions. The first four relate to whether transcription is more dramatically affected by methylation of the region of transcriptional initiation than by elongation. (i) If the promoter region is free of CpG methylation but is surrounded by fully methylated DNA, does the transcriptional activity rise to the level of an entirely unmethylated gene? (ii) If the promoter is the only methylated region, does the transcription level fall to that of a fully methylated gene? (iii) If the structural gene (coding portion) is the only methylated region and is surrounded by unmethylated DNA, is transcription fully suppressed to the level of a methylated gene flanked by methylated DNA? (iv) If the structural gene is the only methylation-free region on the entire minichromosome, is transcription suppressed at all? The two other questions concern the stability of a boundary between a methylated region and an unmethylated region. (v) If a methylated domain is flanked by unmethylated DNA or vice versa, does methylation spread into the unmethylated region or is it lost from the methylated region? (vi) Does transcriptional activity play a role in determining whether methylation spreads into an adjacent region?

MATERIALS AND METHODS

Plasmids. A modified pCLH22 (9) with a *Bam*HI site at the 5′ end of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter was used in this study. Partially methylated constructs were generated by ligating methylated and unmethylated portions of the plasmid together with T4 DNA ligase (Boehringer Mannheim Biochemicals). To construct partially methylated plasmids, the appropriate DNA fragments were purified with a GeneClean kit (Bio 101). Methylation of DNA fragments was achieved by treatment of purified DNA fragments with *Sss*I methylase as described below. Ligation was carried out after completion of the methylation of fragments had been confirmed. After ligation, DNA was extracted with phenol-chloroform and precipitated with ethanol. Ligated DNA was quantitated by *Escherichia coli* transformation in parallel with known amounts of pCLH22.

Nomenclature of plasmids. A superscript minus sign indicates that the portion of the plasmid before the sign is unmethylated, and "me" indicates that the portion of the plasmid in front of this designation is methylated in vitro with a methylase. No specific designation other than me is used when *Sss*I methylase is used to methylate the Cs at all CpG sites. When another methylase is used, the specific site is indicated after the me designation. For example, pCLH22^{meHapII} is methylated with HpaII methylase at all *Hpa*II sites. pCLH22^{-} is unmethylated pCLH22, and pCLH22^{me} is pCLH22 fully CpG methylated with *Sss*I methylase. pCLH22^{-}/LTR^{me} has the 518 bases of the RSV LTR promoter as the only methylated region on the 12.1-kb plasmid (Fig. 1A). pCLH22^{me}/LTR^{-} has the reciprocal methylation pattern of an unmethylated RSV LTR promoter surrounded by the *Sss*I-methylated backbone (Fig. 1B). pCLH22^{-}/Luc^{me} has an unmethylated backbone with a fully methylated luciferase coding region (Fig. 1B). pCLH22^{me}/Luc^{-} contains an unmethylated luciferase coding region and a fully methylated backbone (Fig. 1B).

In vitro DNA methylation. DNA was methylated with *Hpa*II or *Sss*I methylase overnight under the conditions recommended by the manufacturer (New England Biolabs). DNA was extracted with phenol-chloroform and precipitated with ethanol after in vitro methylation. The methylation status was confirmed by digestion with methylation-sensitive restriction endonucleases. pCLH22 has 52 *Hpa*II sites, with none of them located in the promoter region. There are 593 CpG sites on this plasmid, with 26 of them within the promoter region and 107 CpG sites within the 1,875-bp fragment of the luciferase gene.

Cell line and transfection. A derivative of the 293 human embryonic kidney carcinoma cell line, 293/EBNA1, was used for transfection (9). This cell line was grown in Dulbecco's modified Eagle medium with 10% fetal calf serum and penicillin-streptomycin. The calcium phosphate transfection method (9, 27) was used throughout this study. All transfections were done in duplicate or triplicate in each experiment, and all experiments were performed multiple times for confirmation.

Episome recovery and analysis. When transfected cells reached confluence, 2.5% of cells were harvested for luciferase analysis, 2.5% of cells were replated on another 100-mm-diameter plate, and the rest were harvested for plasmid DNA. The hygromycin resistance gene is completely suppressed on fully methylated plasmids. Therefore, all experiments were carried out without any selection for the episomal plasmid regardless of the degree of methylation unless otherwise indicated. In experiments in which transfected cells were selected, hygromycin at 200 μg/ml (final concentration) was used.

Plasmid DNA was harvested from transfected 293/EBNA1 cells by the Hirt method (8). In most experiments, 10% of the DNA from each harvest was digested with *Xba*I to linearize the plasmid and an equal amount of DNA was double digested with *Xba*I and *Hha*I or *Hpa*II to determine the methylation status. In indicated experiments, only *Hha*I or *Hpa*II digestion was carried out without *Xba*I linearization. Digested DNA was fractionated on 1% agarose gels, Southern transferred onto nylon membranes, and probed with the entire plasmid or region-specific probes. Southern blots and autoradiographs were quantitated with a PhosphorImager (Molecular Dynamics and Bio-Rad GS525) or densitometer (Bio-Rad).

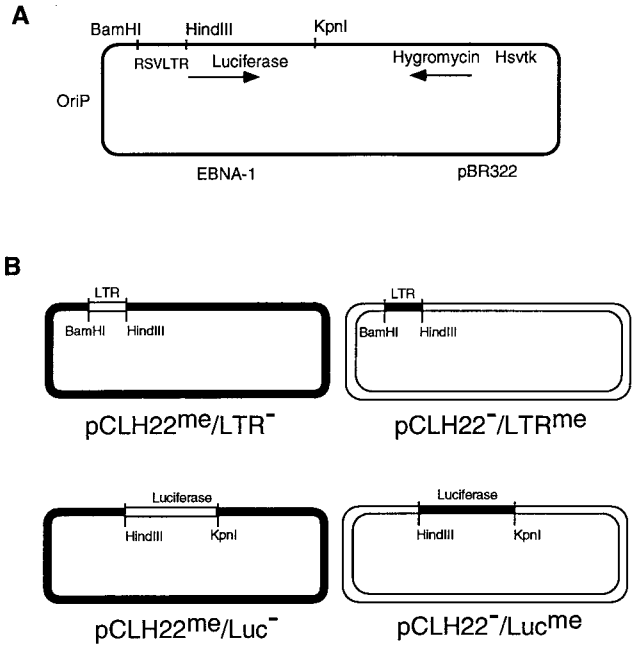

FIG. 1. Construction of partially methylated plasmids. (A) Basic structure of pCLH22. Hsvtk, herpes simplex virus *tk* gene. (B) A wide solid line represents *Sss*I-methylated DNA, and thin parallel lines represent unmethylated DNA. On pCLH22^{me}/LTR^{-}, the RSV LTR is the only methylation-free region of the plasmid. The unmethylated RSV LTR fragment is ligated to the *Sss*I-methylated backbone of the plasmid. The RSV LTR region is the only methylated region on pCLH22^{-}/LTR^{me}; therefore, it has the reciprocal methylation pattern of pCLH22^{me}/LTR^{-}. The RSV LTR fragment is ligated directionally into the backbone with *Bam*HI and *Hin*dIII ends for the construction of pCLH22^{me}/LTR^{-} and pCLH22^{-}/LTR^{me}. The luciferase gene is the only methylation-free region on plasmid pCLH22^{me}/Luc^{-}. On pCLH22^{-}/Luc^{me}, the luciferase gene is the only methylated region; it has the reverse methylation pattern of pCLH22^{me}/Luc^{-}. In pCLH22^{me}/Luc^{-} and pCLH22^{-}/Luc^{me}, the fragment containing the luciferase gene is ligated to the backbone with *Hin*dIII and *Kpn*I ends.

Luciferase expression analysis. An aliquot of transfected cells was harvested and lysed for luciferase activity analysis. Luciferase activities were analyzed on a luminometer (Monolight 2020; Analytical Luminescence) as described previously (11). In this study, the measurement of luciferase gene expression was normalized by the amount of plasmid DNA in cells from each transfection. For each Southern blot (described above), the lowest reading from any lane by PhosphorImager analysis was divided by the reading of each lane to derive the normalization factor for each transfection. The luciferase reading was divided by the normalization factor after subtraction of the background luciferase reading to obtain the normalized luciferase activity. Therefore, the levels of gene expression from the same quantity of plasmid DNAs with different methylation states were compared in this study.

RESULTS

Promoter methylation alone does not significantly suppress transcription. To investigate whether high-density methylation (methylation at all CpG sites) in the promoter can suppress transcription, pCLH22^{-}/LTR^{me}, a plasmid with the promoter as the only methylated region, was used (Fig. 1B) (see Materials and Methods). To compare the impact of methylation, pCLH22^{-}, pCLH22^{me}, and pCLH22^{-}/LTR^{me} were transfected into the 293/EBNA1 cell line. This cell line constitu-

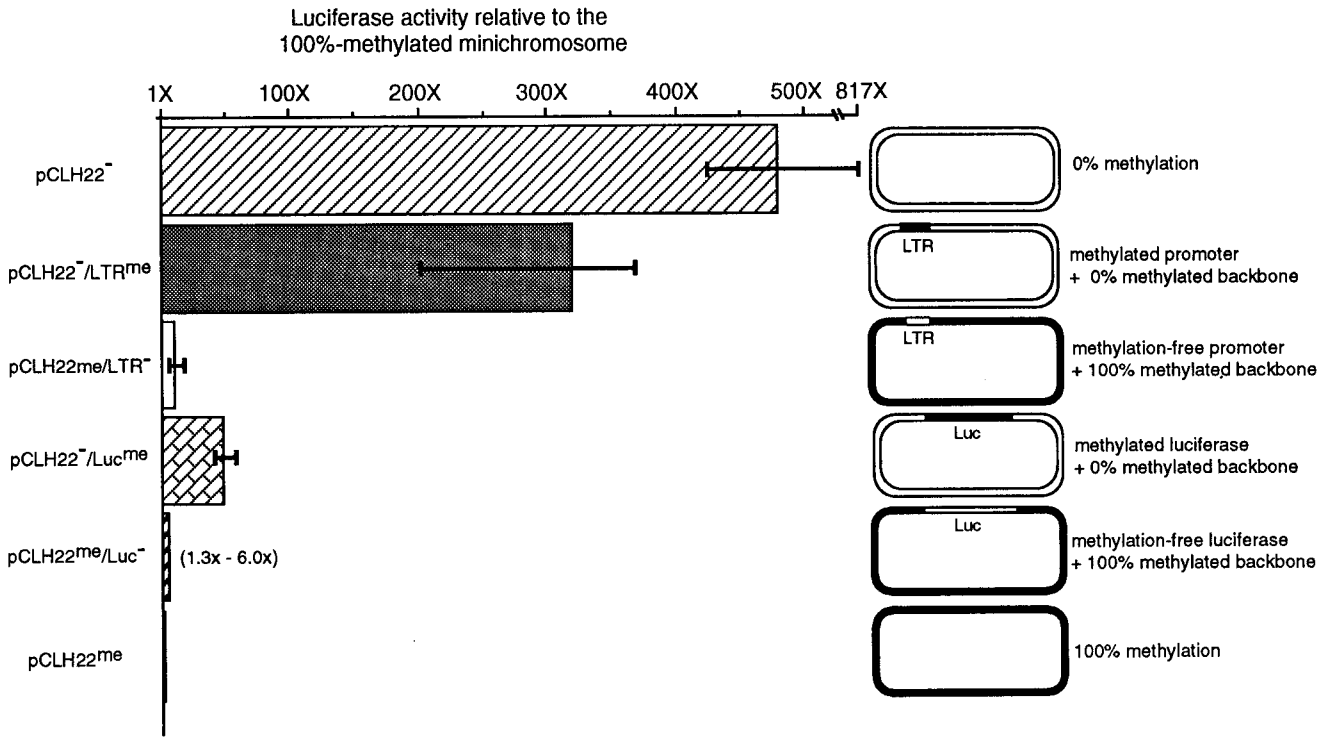

FIG. 2. Transcriptional inhibition and regional methylation. Luciferase expression was normalized for the amount of DNA harvested from cells by Southern blotting. The luciferase activity was normalized first by the quantity of DNA harvested from each transfection. The luciferase activities of all other plasmids presented are relative to that of pCLH22^{me} because the luciferase activity of pCLH^{me} was the lowest. Data are averages of the relative luciferase activities of the indicated plasmids from several transfections. Bars indicate the range of relative luciferase activities for the same plasmid from different transfections. The diagrams to the right of the histogram represent the region of methylation. Solid lines represent *Sss*I-methylated DNA, and thin parallel lines represent unmethylated DNA (Fig. 1). Luc, luciferase gene.

tively expresses EBNA1, thereby permitting the replication of each of these episomes once per S phase.

Luciferase assays and DNA analyses were carried out 8 days after transfection. The luciferase expression of pCLH22^{-}/LTR^{me} was about twofold lower than that of fully unmethylated plasmid pCLH22^{-} (Fig. 2). However, the luciferase expression from pCLH22^{-}/LTR^{me} was more than 200-fold higher than that of the fully methylated minichromosome, pCLH22^{me} (Fig. 2). This clearly demonstrates that high-density methylation in the promoter (26 sites per strand within the 518-bp RSV LTR) does not inhibit transcription significantly when the rest of the minichromosome is unmethylated. This also indicates that CpG methylation does not affect the binding of transcription factors within the RSV LTR. Hence, there is no direct effect of methylation on transcription. It is also clear that a densely methylated region of 518 bp alone is not sufficient to establish transcriptional suppression by some local effect of chromatin structure within that small region. Therefore, the dramatic inhibition of transcription observed in complete minichromosome CpG methylation (9) is not due to an effect of methylation within the region of initiation.

A methylation-free promoter cannot overcome transcriptional suppression caused by the adjacent methylated regions. To examine whether transcription is suppressed when a methylation-free promoter is surrounded by methylated DNA, pCLH22^{me}/LTR^{-}, a plasmid with the promoter as the only methylation-free region, was used (Fig. 1B). pCLH22^{-}, pCLH22^{me}, and pCLH22^{me}/LTR^{-} were transfected into the 293/EBNA1 cell line. Luciferase assays and DNA analyses were carried out as described above. After normalization for the amount of DNA in cells, the luciferase expression from pCLH22^{me}/LTR^{-} was 40- to 50-fold lower than that of the fully unmethylated minichromosome, pCLH22^{-} (Fig. 2). However, luciferase expression from pCLH22^{me}/LTR^{-} remained 8- to 12-fold higher compared to that of the fully methylated minichromosome, pCLH22^{me} (Fig. 2). This indicates that a methylation-free promoter of 518 bp is not sufficient to overcome the transcriptional suppression induced by methylation in the remaining 11.5 kb of DNA. Although the methylation effects on the promoter and the remaining portion of the plasmid appear to be additive (see below), the methylation-free promoter is clearly suppressed by the high-density CpG methylation that surrounds it.

A methylation-free structural gene is not sufficient to establish transcriptional activity. pCLH22^{me}/Luc^{-} (Fig. 1B), which has the luciferase gene as the only unmethylated region on the minichromosome, was used to examine whether inhibition of transcription can be overcome by a methylation-free structural gene. Luciferase assays and DNA analyses carried out 6 days after transfection showed that the luciferase expression from pCLH22^{me}/Luc^{-} was only 1.3- to 6-fold higher than that of the fully methylated minichromosome, pCLH22^{me} (Fig. 2). In the same experiment, the luciferase expression from pCLH22^{me}/LTR^{-} was fourfold higher than that of pCLH22^{me}/Luc^{-} (Fig. 2). It is noteworthy that the 868 bases of simian virus 40 (SV40) poly(A) region downstream from the luciferase gene contain only one CpG site. Therefore, 2,744 bases, including the luciferase coding region and SV40 poly(A) region, on pCLH22^{me}/Luc^{-} contain only one methylated CpG. The luciferase coding region accounts for 15.4% of the minichromosome, and the

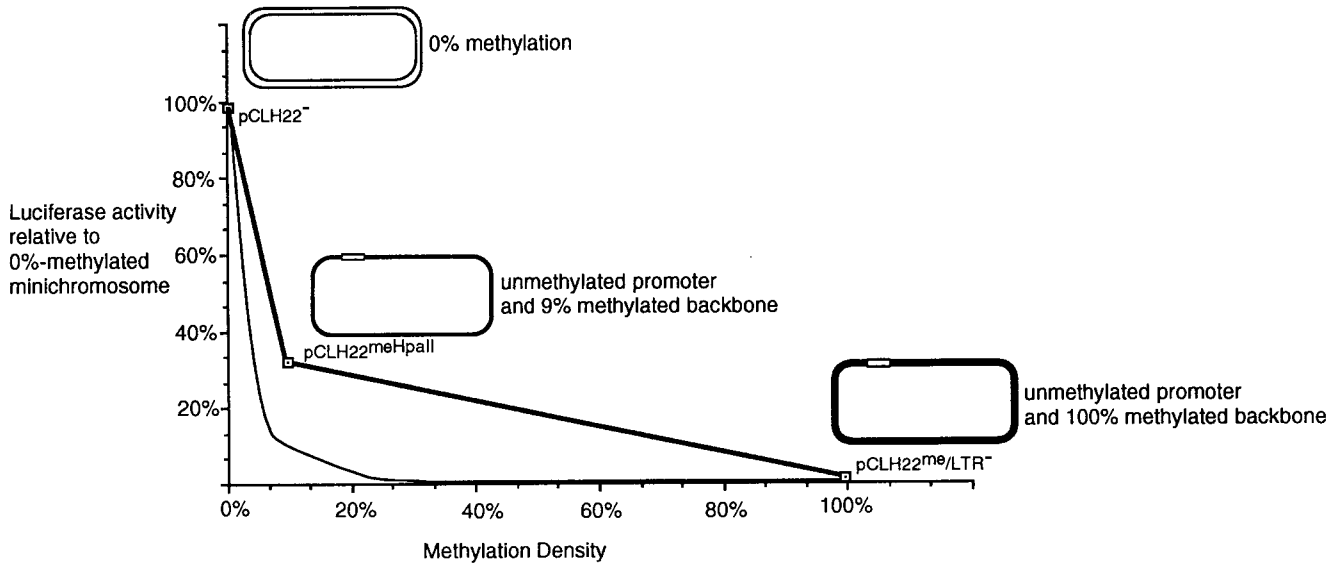

FIG. 3. Transcription inhibition by methylation when the promoter is free of methylation. The *x* axis represents the proportion of methylated CpG sites on the minichromosome. The *y* axis represents the level of gene expression, with that of pCLH22$^-$ set at 100% expression. The luciferase activity was normalized for the amount of DNA harvested from each transfection. A diagram that indicates methylation status is to the right of each datum point, with a wide solid line representing *Sss*I-methylated DNA, a thin solid line representing *Hpa*II-methylated DNA, and parallel thin lines representing unmethylated DNA. pCLH22$^-$, pCLH22^{meHpaII}, and pCLH22^{me}/LTR$^-$ all have fully unmethylated promoters, and 0, 9, and 100% of the CpGs on the remainder of these plasmids are methylated, respectively. For comparison, data for plasmids with 0, 7, 23, and 100% overall methylation (9) are also shown.

luciferase coding region and SV40 poly(A) region together account for 22.5% of the plasmid. Despite the lack of methylation in these regions, transcription from the luciferase gene on pCLH22^{me}/Luc$^-$ failed to occur. The fact that luciferase expression was even more suppressed on pCLH22^{me}/Luc$^-$ than it was on pCLH22^{me}/LTR$^-$ suggests that transcriptional inhibition by methylation is due to the additive effects of backbone methylation and promoter methylation. Otherwise, the minichromosome with the larger unmethylated region (pCLH22^{me}/Luc$^-$) would be expected to show higher expression than that of pCLH22^{me}/LTR$^-$. Considering that the effect of promoter methylation on transcription is small (twofold [see above]), the inability of pCLH22^{me}/Luc$^-$ to express luciferase is predominantly due to global chromatin structure induced by high-density CpG methylation in the backbone that surrounds the luciferase coding region.

Transcription is not abolished by high-density methylation of the coding region. To understand how methylation of only the structural portion of the gene affects transcription, pCLH22$^-$/Luc^{me} (Fig. 1B) was examined. Six days after transfection, the luciferase expression from pCLH22$^-$/Luc^{me} was 10-fold lower than that of the fully unmethylated minichromosome, pCLH22$^-$ (Fig. 2). However, it remained 50-fold higher than that of the fully methylated minichromosome, pCLH22^{me} (Fig. 2). Although transcription was affected by methylation of the 1.9-kb luciferase gene, high-density methylation in the coding region was not sufficient to fully suppress transcriptional activity.

Transcriptional activity is dictated by global methylation. pCLH22^{meHpaII} has an overall methylation density of 9%, but the promoter is free of methylation due to the lack of *Hpa*II sites in this region. With the common feature of a methylation-free promoter, pCLH22$^-$, pCLH22^{meHpaII}, and pCLH22^{me}/LTR$^-$ had 0, 9, and 100% methylation in the remaining 11.5 kb of DNA, respectively (Fig. 3). From a comparison of the transcriptional activities of the luciferase gene from these three plasmids, it was evident that the global density of methylation impacted the transcriptional activity dramatically (Fig. 3). The level of inhibition observed when the promoter region was methylated paralleled the level of inhibition seen previously when the entire minichromosome was methylated (9). This further indicates that the overall methylation density plays a determinant role in transcriptional regulation in this system.

Methylation does not spread into adjacent regions. To examine whether methylation spreads from a small methylated region into an adjacent unmethylated region, pCLH22$^-$/LTR^{me} DNA harvested 8 days after transfection was analyzed by Southern blot analysis. When the *Hha*I sites in the promoter remained methylated, a 2.0-kb *Hha*I fragment was detected when probed with the RSV LTR fragment, and 1.5- and 0.5-kb fragments were detected from unmethylated pCLH22$^-$ (Fig. 4A). As shown in Fig. 4B, the methylation pattern at the *Hha*I sites within the RSV LTR and its adjacent region remained unchanged because no *Hha*I fragment of increased size was detected with the RSV LTR probe. To investigate the possibility that methylation spreads over longer time intervals, an aliquot of transfected cells was subjected to selection with hygromycin. DNA was harvested 40 and 49 days after transfection and analyzed. The *Hha*I sites in the promoter region remained methylated on the majority of plasmids, and a small fraction of DNA showed demethylation within the RSV LTR (Fig. 4C). Furthermore, the restriction fragment size did not reflect any gain of methylation at adjacent *Hha*I sites (Fig. 4C). This indicates that the boundary of methylation in a small region can be maintained for at least 49 days. The same observation was made for pCLH22$^-$/Luc^{me} 8 and 15 days after transfection. The presence of a 2.6-kb *Hha*I fragment indicated that the luciferase gene remained methylated on pCLH22$^-$/Luc^{me}. Otherwise, fragments of 1.1, 0.6, and 0.45 kb would have been detected from the luciferase gene region (Fig. 5A). Other *Hha*I fragments of unmethylated pCLH22$^-$ and fragments from the unmethylated backbone of pCLH22$^-$/Luc^{me} can also be seen when probed with the entire plasmid. As illustrated in Fig. 5B, no loss of methylation at *Hha*I sites

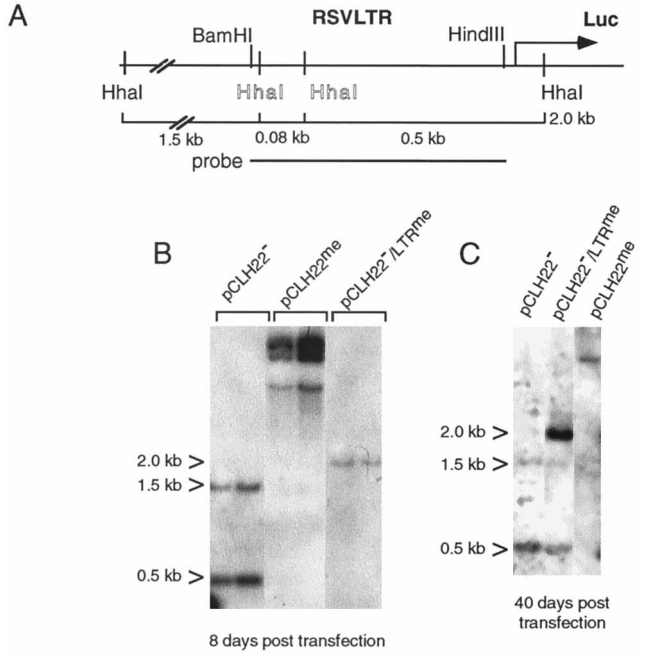

FIG. 4. Methylation does not spread from the RSV LTR into adjacent regions. (A) The diagram illustrates the *Hha*I sites examined in the RSV LTR and surrounding regions. The RSV LTR fragment between the *Bam*HI and *Hin*dIII sites is the only methylated region on pCLH22^{-}/LTR^{me}. Outlined letters indicate methylated *Hha*I sites which are not digested with *Hha*I. Solid letters indicate unmethylated *Hha*I sites which can be digested with *Hha*I. Luc, luciferase gene. (B) Southern blot of DNA harvested 8 days after transfection and digested with *Hha*I. The probe used was the same as that used for panel A. The 1.5- and 0.5-kb *Hha*I fragments were detected from pCLH22^{-} DNA that was unmethylated, and all *Hha*I sites were digested with the enzyme. In contrast, only a 2.0-kb *Hha*I fragment was observed from pCLH22^{-}/LTR^{me} DNA because the two *Hha*I sites within the RSV LTR remained methylated and were not digested with the enzyme. pCLH22^{me} DNA remained undigested by *Hha*I. (C) Southern blot of DNA harvested 40 days after transfection with hygromycin selection. pCLH22^{me} was not hygromycin selected because hygromycin gene expression is suppressed from this plasmid. DNA was digested with *Xba*I and *Hha*I. The observations made in regard to panel B were also true for DNA harvested 40 days after transfection. Some pCLH22^{-}/LTR^{me} DNA became demethylated at the *Hha*I sites within the RSV LTR region; however, most of the DNA remained methylated at both of the *Hha*I sites in this region.

within the luciferase gene region and no gain of methylation at *Hha*I sites surrounding the luciferase gene were observed 8 days after transfection. This observation remained the same for DNA harvested 15 days after transfection (data not shown). These findings suggest that methylation does not spread from a small region into adjacent regions.

I also investigated whether methylation spreads into a small region when this is the only region that is free of methylation on the minichromosome. I analyzed pCLH22^{me}/LTR^{-} DNA harvested 8 days after transfection and assayed the methylation status by Southern blotting. If the RSV LTR region remains unmethylated, a 0.5-kb fragment derived from *Hha*I site at the 3′ end of the RSV LTR region and the *Xba*I site should be detected. As reported previously (9), some preferential demethylation at the *Hha*I site(s) within *oriP* can be observed. This *Hha*I site demethylation resulted in a 1.5-kb fragment, extending from *oriP* to the *Hha*I site at the 5′ end of the RSV LTR region (Fig. 6A). If the RSV LTR region acquires methylation due to spreading, plasmid DNA should only be linearized, with no digestion in the RSV LTR region. Seven days after transfection, the *Hha*I sites in the promoter were clearly unmethylated and resulted in 1.5- and 0.5-kb fragments in addition to the backbone with the entire plasmid as the probe (Fig. 6B). Other than the *Hha*I sites within *oriP*, the remaining portion of pCLH22^{me}/LTR^{-} remained methylated and was not digested with *Hha*I (Fig. 6B). pCLH22^{me}/Luc^{-} DNA harvested 8 and 15 days after transfection was also analyzed for methylation status by Southern blotting. Two major fragments of 0.6 and 0.45 kb, in addition to one larger fragment containing the rest of the plasmid, should be observed if the luciferase gene remains unmethylated and the rest of pCLH22^{me}/Luc^{-} remains methylated. This was the case. Eight days after transfection, no gain of methylation at any *Hha*I site in the methylation-free luciferase gene and no loss of methylation at *Hha*I sites elsewhere on the plasmid were observed (Fig. 5B). The same observations were made with DNA harvested 15 days after transfection (data not shown). These results demonstrate that methylation does not spread from a larger and fully methylated region into the *Hha*I sites in a smaller and unmethylated region.

On pCLH22^{-}/LTR^{me}, the nearest unmethylated *Hha*I site is 145 bases upstream from the first methylated CpG site in the RSV LTR. The nearest methylated CpG is 38 bases upstream from the unmethylated *Hha*I site in the RSV LTR on pCLH22^{me}/LTR^{-}. The nearest methylated CpG is 145 bases from the

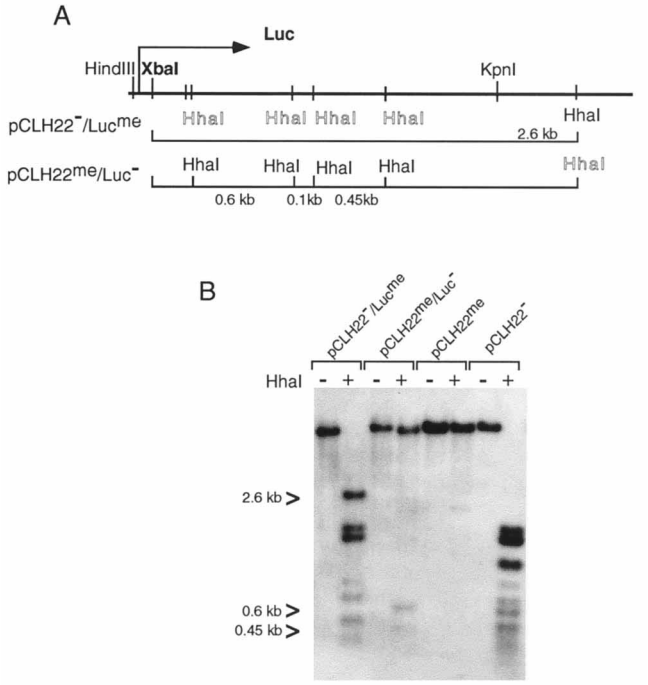

FIG. 5. Methylation does not spread from the luciferase gene into adjacent regions or from adjacent regions into the luciferase gene. (A) The diagram represents the *Hha*I sites examined in the luciferase gene (Luc) and the surrounding regions. The luciferase gene fragment between the *Hin*dIII and *Kpn*I sites is the only methylated region on pCLH22^{-}/Luc^{me} and the only unmethylated region on pCLH22^{me}/Luc^{-}. The methylation status of each *Hha*I site in each plasmid is illustrated with outlined letters for methylated *Hha*I sites and solid letters for unmethylated *Hha*I sites. (B) Southern blot of DNA harvested 8 days after transfection and probed with the entire plasmid. All DNA was digested with *XbaI* to linearize DNA, and *HhaI*-digested DNA was as indicated. pCLH22^{-} DNA was completely digested with *Hha*I due to the lack of methylation. In contrast, a 2.6-kb fragment was detected and 0.6- and 0.45-kb fragments were missing in *Hha*I- and *Xba*I-digested pCLH22^{-}/Luc^{me} DNA. This was due to *Hha*I site methylation within the luciferase coding region. Two fragments of 0.6 and 0.45 kb from the luciferase coding region and an 11-kb fragment from the backbone were detected from *Xba*I- and *Hha*I-digested pCLH22^{me}/Luc^{-} DNA. +, present; −, absent.

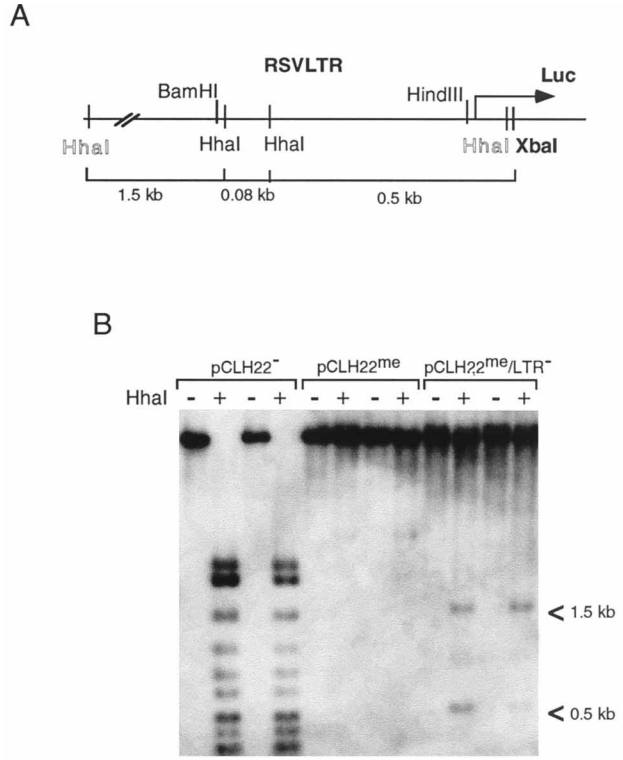

FIG. 6. Methylation does not spread from adjacent regions into the RSV LTR. (A) The diagram illustrates the *Hha*I sites in the RSV LTR and surrounding regions. Outlined letters indicate methylated *Hha*I sites, and solid letters indicate unmethylated *Hha*I sites. Luc, luciferase gene. (B) Southern analysis of methylation status after transfection and probing with the entire plasmid. All DNA was linearized with *XbaI,* and *HhaI* digestions were as designated for panel B. Two bands of 1.5 and 0.5 kb were observed in *Xba*I- and *Hha*I-digested pCLH22^{me}/LTR^{-} DNA. The 0.5-kb band is the *Hha*I-*Xba*I fragment spanning the 3′ end of the RSV LTR and 100 bp of the 5′ end of the luciferase gene to the right of the diagram in panel A, and the 1.5-kb fragment is the result of preferential demethylation in *oriP* and the unmethylated *Hha*I site immediately downstream from the *Bam*HI site. +, present; −, absent.

unmethylated *Hha*I site in the luciferase gene on pCLH22^{me}/Luc^{-}, and the nearest unmethylated *Hha*I site is 451 bases upstream from the first methylated CpG in the luciferase gene on pCLH22^{-}/Luc^{me}. These data show that methylation does not spread into unmethylated *Hha*I sites as near as 38 bases away from a methylated CpG when an unmethylated region of 518 bases is surrounded by 11.5 kb of methylated DNA. This clearly demonstrates that the boundary of the methylated region is quite stable. Moreover, these data also suggest that the methylation pattern of the minichromosome is faithfully maintained over time regardless of the size of the methylated region.

DISCUSSION

This study was designed to answer fundamental questions as to how the dynamic properties of methylation play a role in gene expression. A stable long-term minichromosomal system was used because it mimics the endogenous effects but is free from the positional effects attendant with analysis of endogenous chromosomal genes. Five major findings are described in this study. (i) Initiation of transcription is not blocked by high-density CpG methylation in the promoter (under the circumstance where transcription factor binding is insensitive to CpG methylation at specific sites). (ii) Transcriptional elongation is not eliminated by high-density CpG methylation in a structural gene. (iii) Methylation can repress transcriptional activity in adjacent unmethylated regions. (iv) Transcriptionally inactive chromatin does not predispose adjacent regions to methylation spreading. (v) Transcriptionally active chromatin does not predispose adjacent regions to the loss of methylation.

Transcriptional inhibition by CpG methylation could act at three levels. First, the methylated sequence could have a direct effect; that is, the presence of methyl groups could directly impact transcription factor binding. Second, the local chromatin structure due to the presence of nucleosomes and other proteins recruited by a short patch of methylated DNA might affect transcription. The known recruited proteins include MeCP1 (20), MeCP2 (19), and MDBP-2 (12). The local chromatin structure induced by methylation in the region could either preclude transcription or simply make transcriptional initiation or elongation less efficient because of the competition between the proteins involved in the local chromatin structure and those of the transcription machinery. The third possible mechanism is that a global chromatin structure is initiated in the methylated region but crosses into or through unmethylated regions. Depending on the sizes of the methylated and unmethylated regions, the distance of the methylated region from the transcription unit, and the strength of the promoter, this global chromatin structure could silence adjacent unmethylated regions.

It is essential to assess the effects of methylation on transcription with replicating DNA. It has been described previously that chromatin-mediated transcriptional suppression occurs on replicating plasmids but does not occur on nonreplicating plasmids (1). It has also previously been reported that inhibition of V(D)J recombination occurs at methylated regions of the genome (5) and on methylated replicating episomes but not on methylated nonreplicating episomes (10). These biochemical and transfection studies are in agreement that full suppression of transcription requires replication-dependent chromatin assembly. The inhibition of transcription on the fully methylated minichromosome (compared to that of an unmethylated one) in this system was over 500-fold, whereas the transcriptional repression of fully methylated, nonreplicating plasmids was only 7- (22) and 20-fold (14) in other studies. This 25- to 70-fold disparity between replicating versus nonreplicating minichromosomes clearly indicates that some aspect of the chromatin structure specified by CpG methylation is quite different between replicating and nonreplicating DNAs.

By using a replicating minichromosome in mammalian cells, this study fully supports the view that the global chromatin structure plays a major role in methylation-induced transcriptional inhibition. Regions lacking methylation can be dominated in transcriptional repression by large surrounding regions of methylation. The present study demonstrates that an unmethylated region for elongation, in addition to that for initiation, can be affected by the global chromatin structure induced by methylation in adjacent regions. Although the methylation patch size plays a major role in transcriptional suppression, it is not the sole determinant of the degree of inhibition.

For some promoters, transcription factor binding is affected by the presence of CpG methyl groups, independent of any chromatin effect. This is not the case for the RSV LTR. If transcriptional suppression by methylation is due to the inhibition of initiation by the local chromatin structure, pCLH22^{-}/LTR^{me} should be completely suppressed, whereas pCLH22^{me}/LTR^{-} should have full transcriptional activity. The fact that promoter methylation reduced transcriptional activity by only about twofold suggests that the methylation-induced local chromatin structure impacts transcriptional initiation in only a

minor way. Further, the significant suppression of luciferase transcription from pCLH22^{me}/LTR^{-} indicates that either (i) methylation in the coding region can eliminate transcriptional elongation or (ii) the global chromatin structure induced by methylation in the region surrounding the RSV LTR can affect transcriptional initiation from a methylation-free promoter. The results of experiments in which the coding region was free of methylation (as in pCLH22^{me}/Luc^{-}) suggest that the latter explanation is the primary one (see below).

If the direct effect of methylated sequence or methylation-induced local chromatin structure can eliminate elongation, then pCLH22^{-}/Luc^{me} should have been transcriptionally silent and pCLH22^{me}/Luc^{-} would be expected to retain at least 50% of the transcriptional activity (promoter methylation has only a twofold effect). On the contrary, luciferase expression from pCLH22^{-}/Luc^{me} was reduced only 10-fold compared with that of the fully unmethylated minichromosome and remained 50-fold higher than that of the fully methylated minichromosome. However, luciferase expression from pCLH22^{me}/Luc^{-} was more than 100-fold lower than that of unmethylated pCLH22^{-} and it was only 1.3- to 6-fold higher than that of the fully methylated minichromosome. These findings suggest that transcriptional elongation is not inhibited by methylation itself and that the methylation-induced local chromatin structure is not sufficient to eliminate elongation. It is possible that the local chromatin structure induced by a 1.9-kb gene is sufficient only to slow down transcriptional elongation, not to suppress the process completely. This leads to the question of whether transcriptional elongation can be completely eliminated when the coding region of the gene is larger or the size of the gene relative to that of the entire minichromosome is increased. These questions can also be addressed with this episomal system with different experimental designs. The two lines of evidence mentioned above fully support the conclusion that the global methylation-induced chromatin structure plays an essential role in transcriptional inhibition. Otherwise, the regional effects on the promoter and structural gene should have been much higher than they proved to be.

In general, the replicating stable minichromosome system used here supports the finding of Kass et al. (14) with a transient nonreplicating plasmid that methylation suppresses transcription via the chromatin structure. However, this study demonstrates that promoter methylation may play a minor role instead of no role at all, as proposed by Kass et al. (14). They suggested that the patch size of methylation determines the degree of transcriptional suppression. However, the promoter and structural gene were included as one unit in methylated patches in their analysis. In our study, the luciferase expression of pCLH22^{me}/LTR^{-} was higher than that of pCLH22^{me}/Luc^{-}, even though pCLH22^{me}/Luc^{-} has a methylated patch of DNA of 10.3 kb and pCLH22^{me}/LTR^{-} has 11.6 kb of methylated DNA.

Our preliminary findings at various time points, including 6, 12, 24, and 48 h and later after transfection (data not shown), suggest that transcriptional inhibition by methylation is most likely time dependent in this replicating minichromosomal system. Using a nonreplicating plasmid in the *Xenopus* oocyte system, Kass et al. (15) also observed time-dependent transcriptional inhibition to some extent. In the *Xenopus* oocyte system, cell cycle and cell population effects are not relevant. In addition, replication of double-stranded DNA does not occur. In contrast, the mammalian system has more complex factors involved. First, the entire cell population is not likely to be in the same stage of the cell cycle upon DNA entry. Therefore, a plasmid may replicate in some cells before it does in others. Second, methylated plasmid DNA may have different replication timing compared with that of unmethylated DNA just as the methylated portion of the endogenous genome does (the efficiencies of replication were similar for methylated and unmethylated plasmid DNAs [10]). Third, transcription inhibition can be due to additive effects of the chromatin structure specified by methylation and replication. Methylation and replication have independent and synergistic effects on endonuclease accessibility (10), which appears to have its basis in the chromatin structure. All of these issues must be investigated in order to definitively address the extent to which transcriptional inhibition by DNA methylation in mammalian cells is time dependent. These questions can be addressed by using the replicating minichromosomal system, and I am in the process of doing so.

A recent study by Nan et al. (22) showed that a patch-methylated adenovirus major late promoter inhibited transcription about fourfold and that methylation in the regions surrounding the promoter inhibited transcription about twofold in an in vitro transcription assay with added methylation binding protein MeCP2. Considering the likely lack of a fully assembled chromatin structure in the in vitro system, the fourfold inhibition by promoter methylation observed may have been due to the effect of methylation at specific sites instead of the chromatin structure. Furthermore, the lack of significant impact on transcription by methylation other than in the promoter in the study by Nan et al. (22) suggests that MeCP2 alone without chromatin structure is not sufficient to establish full transcriptional inhibition. Therefore, the significant inhibition of transcription observed for pCLH22^{me}/LTR^{-} (40- to 50-fold) is most likely due to proteins involved in the chromatin structure in addition to MeCP2. Alternatively, the full transcriptional inhibition seen here may require the binding of MeCP2 and possibly some other proteins recruited by CpG methylation as the chromatin structure assembles during replication. The evidence provided here and in studies described above strongly suggests that methylation of the promoter and structural gene each plays a minor role in transcriptional inhibition and that the chromatin structure has the dominant impact on transcription. However, the true impact of methylation patch size on transcription remains unanswered and needs to be evaluated by assessing segments other than the promoter and reporter gene for comparison. I am designing experiments to address this question by methylating regions away from the promoter and structural gene on the minichromosome to investigate how the patch size, the distance of the methylated patch from the reporter gene unit, and the density of the methylated patch influence transcription of the reporter gene.

In the present study, the spreading of methylation into adjacent regions was not observed despite many rounds of replication (7, 15, 40, and 49 days after transfection). The region of methylation was faithfully maintained regardless of the size of the region and the extent of transcription suppression. When only the 518 bp of the RSV LTR was methylated, the transcription remained active and methylation did not spread into adjacent unmethylated regions. One can argue that transcriptional activity prevents methylation spreading. However, methylation did not spread into the RSV LTR when the RSV LTR of 518 bases was the only methylation-free region on the minichromosome and transcriptional activity was reduced significantly. The same observations were made for the 1.9-kb luciferase gene. These findings demonstrate that methylation does not spread regardless of transcriptional activity or patch size of methylation. This suggests that inactive chromatin is not likely to be the cause of de novo methylation observed in integrated (26) or episomal (6, 21) EBV DNA or at sites in the genome. Although it is possible that the integrated or genomic

sequences and episomal DNA are sequestered in different nuclear compartments, it is highly unlikely that episomal EBV DNA is in a nuclear compartment different from this *oriP*-based minichromosome. Therefore, the DNA sequence itself or the sequence-induced local DNA structure may play an important role in targeting de novo methylation. In the future, this long-term stable episomal system can be used for studies that involve de novo methylation and demethylation.

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