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Genetics

Cellular and viral DNA hypomethylation associated with induction of Epstein–Barr virus lytic cycle

(Epstein–Barr virus DNA demethylation/Epstein–Barr virus amplification/early antigen and viral capsid antigen production/inhibition by retinoic acid)

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ABSTRACT **Epstein–Barr virus (EBV) producer and nonproducer cell lines have been treated with a combination of phorbol 12-myristate 13-acetate and *n*-butyrate (sodium salt). These inducers caused a massive hypomethylation of the EBV producer line P3HR-1 DNA (about 30%) at the time when DNA replication was inhibited. The viral DNA in these cells is heavily methylated as judged by digestion with *Hpa* II and probing with the *Bam* HI H fragment of EBV. However, upon induction with phorbol 12-myristate 13-acetate and *n*-butyrate, total hypomethylation of this viral DNA region was observed within 24 hr. This hypomethylation preceded EBV amplification, which became apparent only 32–36 hr after induction. When induction was carried out in the presence of retinoic acid, hypomethylation of cellular and viral DNA, viral DNA amplification, and production of the viral early antigen and viral capsid antigen were substantially inhibited. EBV DNA in another producer line (Jijoye nude) and in the nonproducer line Raji was hypomethylated and did not undergo further hypomethylation in response to induction. The observed hypomethylation of P3HR-1 and EBV DNA in the absence of DNA replication suggests that it is achieved by an active demethylation mechanism. This changes our perception of the DNA methylation phenomenon, since it has been generally accepted that hypomethylation of DNA takes place by a passive mechanism that involves DNA replication in the absence of methylation.**

Tissue-specific DNA methylation patterns have been shown to exist in eukaryotic cells (1). The nature of the events leading to the establishment of DNA methylation patterns, presumably during differentiation, is still obscure. It generally has been accepted that these patterns are formed in the early embryo by a local change directed by site-specific inhibition of the methylation reaction (2, 3). However, it has been shown recently that the process of differentiation of mouse teratocarcinoma cell lines (4, 5) and Friend erythroleukemia cells (6) is associated with genome-wide changes in the extent of DNA methylation. A closer insight into the mechanisms involved in the changes in DNA methylation during differentiation requires a better understanding of major cellular events. Therefore, we have set out to study this question in a well-defined inducible eukaryotic system.

Human lymphoid lines harboring the Epstein–Barr virus (EBV) regularly express the nuclear antigen EBNA, while expression of the lytic early antigens (EA) and viral capsid antigens (VCA) is suppressed in proliferating cells (7). In virus producer lines, 0.5–5% of the cells are spontaneously producing virus at any given time (8). Lines that show some spontaneous viral antigen production may be induced to enter the viral cycle by chemical agents, such as *n*-butyrate or phorbol 12-myristate 13-acetate (PMA), which are known to induce differentiation in other systems (9–11). Some observations on the involvement of DNA methylation in the induction of the EBV lytic cycle have been made previously. Ben-Sasson and Klein have shown that 5-azacytidine (a DNA methylase inhibitor) activated the EBV lytic cycle in latently EBV-infected lines (12). Hypomethylation of EBV DNA fragments after induction has been reported as well (13–16). However, no attempt has been made to study EBV DNA methylation by appropriate kinetic studies. Such kinetic studies can throw some light on the targets within the cell that are affected by the inducers and the role played by the changes in DNA methylation in the process of the induction.

The EBV producer cell line P3HR-1 has been used for the present study. Some other cell lines have been analyzed as well. The kinetic experiments show that genome-wide hypomethylation that precedes virus expression and amplification is caused by the inducers.

MATERIALS AND METHODS

Cell lines were grown in RPMI 1640 medium (GIBCO) supplemented with 5% fetal calf serum and antibiotics. P3HR-1 (17), Raji (18), and Jijoye nude (19) are EBV-positive Burkitt lymphoma lines. PUTKO-1 (20) is a hybrid between K-562 (21), a cell of myeloid lineage, and P3HR-1.

Induction experiments were always performed on confluent cell cultures. At the time of induction, the cells were diluted to 0.5×10^6 cells per ml in fresh culture medium and inducer(s). The cells were incubated at 37°C until the time of harvest. PMA (Sigma) was used at a final concentration of 10 ng/ml; *n*-butyrate (sodium salt; Sigma) at 3 mM; and retinoic acid (Sigma), at 10 μM.

Immunofluorescence (22) has been performed on smears fixed in acetone/methanol, 2:1 (vol/vol). The cells were stained with fluorescein isothiocyanate-conjugated antiserum "Jeremia" against the EBV EA or the antiserum "Buya" against the VCA; 500 cells were counted on each slide.

Thymidine Incorporation. PMA- and butyrate-induced cells and untreated controls were collected at the times indicated in Fig. 1. Cells (10^6) were resuspended in 1 ml of fresh medium containing 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (New England Nuclear) and also PMA/butyrate if the cells were previously exposed to these chemicals. After 1 hr of incubation at 37°C, the cells were harvested onto Whatman GF/C filters. The filters were dried and assayed in a LKB rack β liquid scintillation counter. Values represent triplicate measurements.

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Abbreviations: EBV, Epstein–Barr virus; EA, early antigen(s); VCA, viral capsid antigen(s); PMA, phorbol 12-myristate 13-acetate; kb, kilobase(s).

Analysis of the Extent of DNA Methylation at CpG Sequences. The degree of methylation of CpG-containing sequences was determined by a previously described method (23, 24). According to this method, DNA samples were nicked with DNase I (Sigma), and the 3' ends of the nicks were end-labeled with [α-^{32}P]dGTP (Amersham, 3000 Ci/mmol) by using *Escherichia coli* DNA polymerase I (Boehringer Mannheim). The labeled DNA was digested to deoxyribonucleoside 3'-monophosphates by using spleen phosphodiesterase and micrococcal nuclease. The digest was chromatographed in two dimensions on cellulose-coated TLC sheets (Eastman-Kodak). The chromatograms were autoradiographed, and the autoradiograms were scanned with a Helena Laboratories (Beaumont, TX) Quick Scan.

RESULTS

Induction of the EBV Lytic Cycle Is Associated with Cellular DNA Hypomethylation. As a first step toward the elucidation of the involvement of DNA methylation in the EBV induction process, we tested the possibility that induction of the lytic cycle of EBV by known inducers of differentiation is associated with a genome-wide DNA hypomethylation. EBV producer and nonproducer cell lines and somatic hybrids were analyzed for the extent of methylation of cellular DNA before and after induction by PMA/butyrate. Purified DNA samples from the various cells were subjected to a modified nearest-neighbor analysis of the state of methylation at the dinucleotide CpG sequences (23). Spontaneous or induced activation of the viral cycle leads to the sequential appearance of viral proteins, EA and VCA. Therefore, the induction process was monitored by scoring EA- and VCA-positive cells identified by immunofluorescence as previously described (22). Induction of the P3HR-1 producer cell line by butyrate and by PMA/butyrate involved a loss of 20–30% of the methyl moieties in the cell genome by 48 hr after induction (Table 1). The synergistic effect of PMA/butyrate should be noted. Such an effect also has been observed recently in FELC cells (6).

Jijoye nude cells, which undergo less-efficient induction, also undergo a lower level of hypomethylation (10%). Other cell lines that exhibit low levels of inducibility do not show any detectable hypomethylation (±2%). The nonproducer cell line Raji may be induced to a relatively low level of expression of the EA antigen, but no VCA is made, and the system fails to proceed to the lytic cycle in response to chemical induction (17). This line did not undergo a significant change in the extent of its cellular DNA methylation. However, it should be emphasized that the level of its DNA methylation (before treatment) was about 10% lower than that observed in the P3HR-1 line (Table 1).

Table 1. DNA methylation and expression of the genes for EA and VCA in P3HR-1 and Raji cells

Cell line	Treatment	Antigen positive, % of total cells		CpG methylation, % of total CpG
		EA	VCA	
P3HR-1	Control	2	1 ± 0.9	78 ± 2
	PMA	16	6	78 ± 2
	Butyrate	40 ± 10	35 ± 5	65 ± 2
	PMA/butyrate	45 ± 12	35 ± 5	52 ± 7
Raji	Control	0	0	70 ± 2
	PMA	5.3	0	69 ± 3
	Butyrate	5	0	70 ± 2
	PMA/butyrate	25	0	68 ± 2

Human lymphoma cell lines harboring EBV were treated with 10 ng of PMA or 3 mM butyrate or both for 48 hr. The percentage of EA- and VCA-positive cells was determined by counting cells on acetone/methanol-fixed smears, stained by direct immunofluorescence as described (22). DNA samples were prepared from treated and untreated cells, and the extent of DNA methylation at CpG sites was determined by nearest neighbor analysis as described. Each value represents an average of three determinations.

As the level of induction is lower in Raji cells than in P3HR-1, the degree of hypomethylation may not be detected in the same way as in P3HR-1. An alternative explanation may be that induction of EA in Raji cells operates via a mechanism that is independent of any further hypomethylation. This is in accordance with the observation that Raji cells were not induced by 5-azacytidine, whereas P3HR-1 cells were induced after 5-azacytidine treatment (12). It is tempting to speculate that induction is a multistep process that involves hypomethylation among other events. Raji cells may have undergone the hypomethylation event at a previous stage, as suggested by their lower basal level of methylation, but lack another essential factor.

Another interesting line is PUTKO-1, a somatic hybrid between P3HR-1 and K-562 (a human erythroleukemia cell line). This hybrid is an absolute nonproducer. K-562 was distinct in its low level of DNA methylation (50%) (most mammalian somatic tissues are methylated at about 80% of the CpGs). The extremely low level of DNA methylation dominated in the cell hybrid PUTKO-1 (45%) and was associated with other K-562 phenotypic characteristics. This suggests that the methylation capacity of a cell may be an inherited trait.

Hypomethylation may be achieved by a passive mechanism that involves inhibition of the maintenance methylation reaction during DNA replication or by an active mechanism of unknown character. To determine which of these two possible mechanisms operates, we followed the kinetics of cellular DNA replication after induction. It previously has been shown that induction of the EBV lytic cycle involves arrest of cellular DNA replication (25). This experiment was repeated, and the results are shown in Fig. 1. Inhibition of cellular DNA replication could be detected 16 hr after induction and reached its peak at 36 hr. Thus, surprisingly, the hypomethylation event was associated with a period of arrest in DNA replication, suggesting an active demethylation mechanism.

Pattern of Methylation of Viral *Bam*HI H Fragments. At this stage the ground was ready for an analysis of the events that accompany induction at the gene level. The fragment that was chosen for this study is the 4.3-kilobase (kb) *Bam*HI EBV H fragment (in P3HR-1 cells), which has been shown to code for the EA-R region of the EA complex (26). This analysis was aimed at elucidating the pattern of methylation that characterizes the nonproductive stage and trace the changes that occur after exposure of the cells to inducers. In order to gain a more general view, a number of producer and nonproducer cell lines were analyzed.

The 4.3-kb *Bam*HI H fragment contains numerous CpG sites, which may be probed by *Hpa* II and *Msp* I restriction enzyme analysis. A complete digestion of this fragment with *Msp* I, which is not inhibited by CpG methylation, resulted in five distinct groups of bands representing fragments in the size range of about 1.0, 0.66, 0.55, 0.2, and 0.14 kb, respectively (Fig. 2*E*). Methylation of all or part of the CpCpGpG sites should have resulted in a different set of bands. A precise analysis of the location of the unmethylated sites is a formidable task in the light of the fact that many sites are clustered, generating fragments of extremely small size (10–100 base pairs). However, interesting conclusions could be drawn from scans of the banding pattern of the *Hpa* II digests. As is shown in Fig. 2, the methylation pattern of the *Bam*HI H fragments varied widely in the various cell lines. Three groups of fragments could be observed: fragments in the range of 3–4.3 kb, which denote extensive methylation of most sites and site-specific hypomethylation of a small

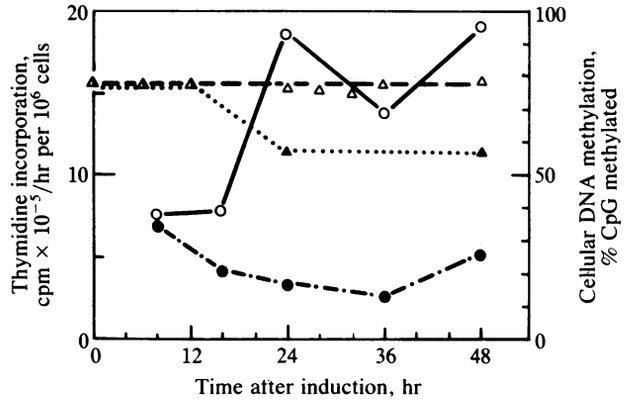

FIG. 1. DNA synthesis and extent of CpG methylation in P3HR-1 cells and cells induced with PMA/butyrate. P3HR-1 cells were grown and treated with PMA/butyrate as described. Total cellular DNA synthesis in the P3HR-1 cell line was measured by pulse-labeling 10^6 cells with [^{3}H]thymidine (1 μCi) for 60 min. ○, Untreated controls; ●, cells treated with PMA/butyrate. The extent of CpG methylation was determined as described. △, control cells; ▲, cells treated with PMA/butyrate.

number of sites; fragments in the range of 1.3–2.6 kb, which result from a more widespread hypomethylation; and low molecular weight fragments that are identical to the *Msp* I pattern and represent essentially unmethylated molecules.

In the P3HR-1 producer cells, two distinct populations of fragments could be discerned. Most of the molecules (≈80%) were heavily methylated, with two or more specific unmethylated sites indicated by the appearance of two distinct fragments of 3.3 and 2.6 kb, which are smaller than the expected 4.3-kb *Bam*HI fragment (Fig. 2*A* and Fig. 3*A*). At the same time, some molecules were essentially unmethylated as judged by the presence of the small fragments that correspond to the *Msp* I banding pattern (see Fig. 3*A*). Expression occurred only when about 95% of the molecules were unmethylated. These observations may correlate with the state of expression of EA and VCA antigens in the cells before and after their exposure to the inducer (Table 1). Another producer cell line, Jijoye nude, was induced with less efficiency; however the *Bam*HI H region was hypomethylated to a large extent in the uninduced state as well (Fig. 2*B*). This suggests that undermethylation of the region coding for EA is not sufficient for EA expression. In Raji cells viral molecules were less methylated than in P3HR-1, as judged by the appearance of lower molecular weight fragments (2.6, 1.1, and 0.8 kb). Very little change in the pattern of methylation occurred after induction of the cells (Fig. 2*D*). This correlates well with low levels of cellular DNA methylation and failure of the inducers to cause further hypomethylation of genomic DNA (Table 1). Thus, the expression of EA in these cells does not involve any further event of hypomethylation. In another nonproducer cell, PUTKO-1, the virus was also partially methylated, and very little change took place upon induction (Fig. 2*C*). It should be recalled that PUTKO-1 is a somatic hybrid of P3HR-1 and K-562 and that the EBV derives from the P3HR-1 parent after somatic hybridization. The methylated molecules underwent partial undermethylation, as judged by the appearance of the 0.8-kb and 0.62-kb fragments. The partial undermethylation in PUTKO-1 may reflect the low methylation capacity of the host (45% of the CpGs are methylated; *vide supra*).

Viral Hypomethylation Precedes Viral Amplification. The results of analysis of the *Bam*HI H state of methylation, as described above, may suggest that the lytic state of the virus in producer cells is characterized by undermethylation of the entire viral *Bam*HI H region. The nonlytic stage may not be

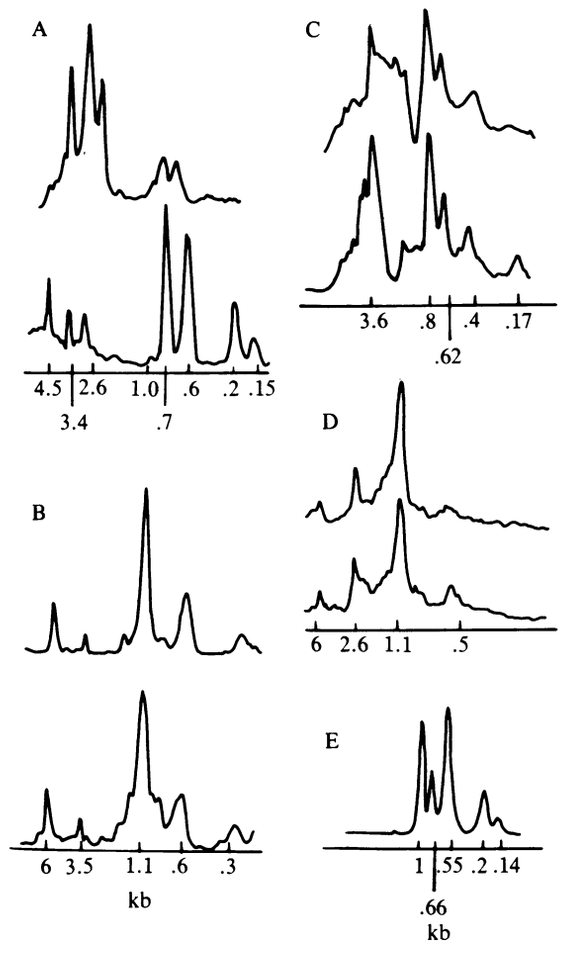

FIG. 2. The pattern of DNA methylation at CpCpGpG sites of *Bam*HI H fragments of induced and uninduced EBV producers and nonproducer cell lines. DNA was purified from various induced (treated for 48 hr with PMA/butyrate) and noninduced EBV producers and nonproducer cell lines. The DNA was digested with *Bam*HI and *Hpa* II. DNA fragments were fractionated by electrophoresis on 1% agarose blotted to nitrocellulose sheets and hybridized to ^{32}P-labeled *Bam*HI H fragment of EBV. The autoradiograms were scanned by using Helena Laboratories Quick Scan. Size markers were *Hae* III ϕX174 fragments and *Hin*dIII λ phage fragments. (*A–D*) *Bam*HI/*Hpa* II-digested DNA from P3HR-1 (*A*), Jijoye nude (*B*), PUTKO-1 (*C*), and Raji (*D*). (*E*) *Bam*HI/*Msp* I-digested. Each upper trace is of nontreated cells, and the lower trace represents digested DNA from 48-hr treated cells.

defined by a characteristic pattern of methylation. Different levels of partial methylation may exist that do not correlate with the capacity of the cell to be induced. The final methylation pattern may be a result of transcription and/or amplification. To test these possibilities, detailed kinetics of the loss of methyl groups in the *Bam*HI H fragment, EBV expression (EA and VCA production), and viral amplification have been carried out in P3HR-1 cells upon induction with PMA/butyrate. Viral amplification has been measured by dot-blotting cellular DNA and hybridization with an α-^{32}P-labeled *Bam*HI H fragment. The results presented in Fig. 3 clearly show that the process of viral hypomethylation was accomplished by 24 hr (Fig. 3*A*). This was before EA and VCA appeared (at 24 hr) (Fig. 3*C*) and well before viral amplification started at 32–36 hr after induction (Fig. 3*B*). It should be noted that no amplification was observed till 24 hr. These results, taken together with the kinetics of hypomethylation at the cellular genome level (Fig. 1), support the

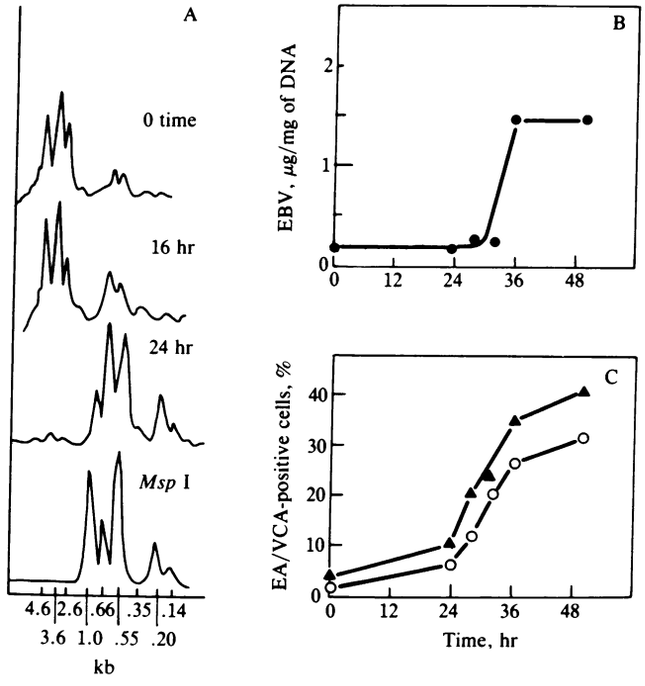

FIG. 3. Kinetics of viral hypomethylation and viral amplification and expression after induction with PMA/butyrate. P3HR-1 cells were treated with PMA/butyrate as described in the text. Cell samples were removed at various times after induction. (*A*) EBV demethylation. Total DNA was purified and digested with *Bam*HI and *Hpa* II (three upper scans) or *Bam*HI and *Msp* I restriction enzymes. The cleaved DNA samples were electrophoresed, blotted, and hybridized to α-^{32}P-labeled *Bam*HI H probe. Autoradiograms were scanned and size markers were as described in the legend to Fig. 2. (*B*) EBV amplification. DNA samples (50 ng) were dot-blotted on nitrocellulose filters and hybridized to a α-^{32}P-labeled *Bam*HI H probe (26). The concentration of EBV sequences was determined by measuring the intensity of the radioactive spots relative to the intensity of a *Bam*HI H standard and expressed as μg of EBV per mg of cell DNA. (*C*) EBV expression. The % EA (▲)- and VCA (○)-positive cells has been determined as described in the methods.

conclusion that the hypomethylation is accomplished by an active mechanism.

Retinoic Acid Inhibits Hypomethylation and Induction. In order to further establish our findings that induction of the lytic EBV cycle by differentiating agents in producer cells is associated with both viral and cellular genome-wide hypomethylation, we used retinoic acid, a known differentiating agent that partially inhibits induction by PMA/butyrate in P3HR-1 cells (Table 2). Addition of retinoic acid to the cells simultaneously with PMA/butyrate resulted in partial inhibition of both cellular and viral hypomethylation, as shown in Fig. 4 and Table 2. Viral amplification and production of EA and VCA antigens were inhibited as well. The pattern of methylation of DNA in cells treated with the combination of retinoic acid, TPA, and butyrate disguised a change in the ratio of methylated to unmethylated cell populations. No partial changes in methylation could be detected, as judged by the appearance of no other fragments. This may suggest that hypomethylation of viral *Bam*HI H fragment after induction is an "all or none" phenomenon. Retinoic acid diminishes the number of cells that undergo induction (Table 2) but may not change the sequence of events in cells that were already committed to the lytic cycle.

DISCUSSION

The present study demonstrates that induction of the lytic EBV cycle is associated with both genome-wide hypomethylation and hypomethylation of viral gene sequences. The hypomethylation event seems to precede production of viral antigens and definitely precedes viral amplification (Fig. 3). Moreover, the cellular DNA hypomethylation occurs at a time when the replication is inhibited (Fig. 1). Thus, hypomethylation cannot be regarded as a result of replication or transcription. However, when the effect of PMA/butyrate is inhibited by retinoic acid, cellular and viral hypomethylation is inhibited as well. This suggests that hypomethylation is tightly associated with viral induction.

The results presented here lend support to a different perception of the mechanism of hypomethylation (6). We demonstrate here that eukaryotic cells possess a mechanism of overall hypomethylation that may be triggered by various chemical inducers. Hypomethylation of the induced gene correlates with the overall hypomethylation of the cellular genome. When no cellular hypomethylation occurs, as is the case with the nonproducer cell lines, gene hypomethylation is not taking place. The same is true for retinoic acid-treated producer cells.

We suggest that the inducing agents trigger a cellular mechanism for hypomethylation and do not act by directly inhibiting the methylase during cellular DNA replication. This argument is supported by the fact that hypomethylation is induced late after addition of the chemicals and that addition of the same chemicals to other cells, such as Raji, although effective in other respects, is unable to induce hypomethylation. Moreover, retinoic acid, which has been shown to induce hypomethylation in teratocarcinoma cell lines (4, 5), inhibits hypomethylation in P3HR-1 cells (Table 2). All of these observations are in accordance with a model that suggests that the hypomethylation mechanism is operated by the network of the intensively studied intracellular messenger systems (27). The state of cell differentiation may determine whether an extracellular signal will trigger hypomethylation or not. Thus, the same inducers may trigger hypomethylation in some cells and inhibit it in others. The fact that induction is associated with replication arrest and that hypomethylation occurs late after induction makes unlikely the commonly accepted model that hypomethylation is achieved by a passive mechanism mediated by inhibition of DNA methylation during replication. Recent observations in

Table 2. Effect of retinoic acid (RA) on induction of EA and VCA and on DNA methylation

Treatment	Antigen positive, % of total cells		Viral amplification, pg/ng of host DNA	CpG methylation, % of total CpG
	EA	VCA		
Control	2	1.7	0.1	76
RA	2	2	0.3	74
PMA/butyrate	57	42	3	53
PMA/butyrate/RA	21	29	2	63

Cells were treated and CpG methylation and EA and VCA production were determined as described in the text. Viral amplification was analyzed as described in the legend to Fig. 3.

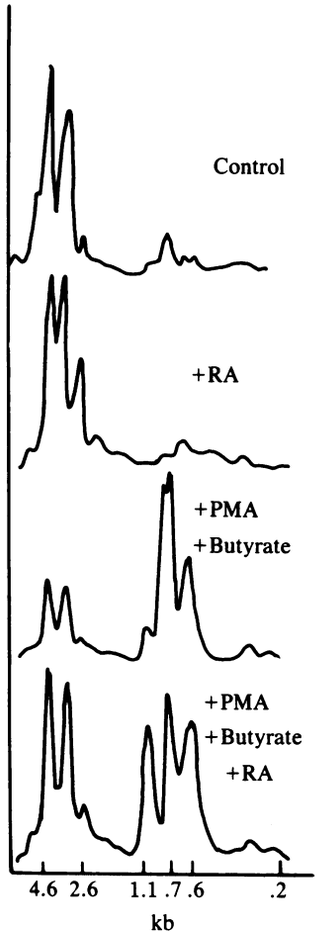

FIG. 4. Effect of retinoic acid on hypomethylation of the *Bam*HI H fragment region. DNA was prepared from untreated P3HR-1 cells (control), cells treated for 48 hr with retinoic acid (+RA), with PMA/butyrate, or with RA/butyrate/PMA, digested with *Bam*HI/Hpa II, blotted, hybridized to α-^{32}P-labeled *Bam*HI H fragment, and autoradiographed. The autoradiogram was scanned by densitometry, and size markers were as described in the legend to Fig. 2. Cell samples were removed at 0 time and at 48 hours after induction.

Friend erythroleukemia cells indeed suggest that hypomethylation may occur without cellular DNA replication (6). Also one methylated site in the 5′ region of the chicken vitellogenin gene has been shown to undergo active demethylation in the absence of replication (28).

The present study may initiate a different approach to the investigation of the role of DNA hypomethylation in the induction of EBV and in cell differentiation in general. The observed genome-wide hypomethylation may be a simple mechanism to hypomethylate viral genes; however, undermethylation of the *Bam*HI H fragment *per se* is not sufficient for EA induction. Alternatively, the general hypomethylation may be the important event in changing the state of differentiation of the cell; this, in turn, may alleviate a cellular regulation on EBV induction.

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