MOLECULAR AND CELLULAR BIOLOGY, Apr. 1988, p. 1443–1448
0270-7306/88/041443-06$02.00/0
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Vol. 8, No. 4

Hypomethylation of Host Cell DNA Synthesized after Infection or Transformation of Cells by Herpes Simplex Virus

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Received 22 July 1987/Accepted 24 December 1987

Infection of rat embryo cells with herpes simplex virus type 2 caused undermethylation of host cell DNA synthesized during infection. DNA made prior to infection was not demethylated, but some of its degradation products, including methyl dCMP, were incorporated into viral DNA. The use of mutant virus showed that some viral DNA synthesis appears to be required for the inhibition of methylation. Inhibition of methylation cannot be explained by an absence of DNA methyltransferase as the activity of this enzyme did not change during the early period of infection. Inhibition of host cell DNA methylation may be an important step in the transformation of cells by herpesviruses, and various transformed cell lines tested showed reduced levels of DNA methylation.

It has been recognized since 1971 (12) that herpes simplex virus type 2 (HSV-2) causes cells to be transformed to an oncogenic phenotype. However, multiple studies on the actual method of transformation have produced a variety of possible properties of the virus which could be involved in transformation. These features include the structure of the transforming DNA involving the presence of insertion sequences (16) or altered repeat elements (22), biological carcinogenesis such as mutation or gene amplification (35, 39), or even the involvement of the ribonucleotide reductase gene whose large and small subfragments map within the DNA sequences involved in the induction of morphological transformation (reviewed by Macnab [26]). What is reasonably clear is that viral DNA encoding a transforming protein is not retained in the cells (9, 15).

Our interest in the induction of morphological transformation continues from our findings that transformed cells all react with antisera raised against HSV-infected cells (9, 26, 27). The results demonstrate that infection with HSV induces the expression of immunologically competent cell polypeptides whose expression can also be detected in transformed cells. These polypeptides are expressed at low levels throughout the cell cycle in uninfected cells (N. B. LaThangue, personal communication) but are increased on HSV infection and in transformed cells.

Alteration in the expression of cell polypeptides can occur as a result of gene amplification, and cellular gene amplification has been reported to occur after HSV transformation (28, 30, 31, 39; J. C. M. Macnab, unpublished results).

Tumor cells can have a reduced level of DNA methylation when compared with normal control cells (5, 13, 14, 24, 25; reviewed in reference 2), and Gama-Sosa et al. (17) found that the level of methylation decreases on going from benign, through primary malignant, to metastatic tumors. Treatment of cells with carcinogens is also known to cause an inhibition of DNA methylation (6, 34), and this may be directly involved in carcinogenesis. The nonspecific demethylation of DNA may lead to the chance activation of a cellular gene involved in cell transformation. Indeed, Hsiao et al. (18) have shown that treatment of mouse embryo fibroblasts with the demethylating agent 5-azacytidine can lead to cell transformation.

In this communication, we present evidence for the hypomethylation of DNA in a number of cell lines derived from foci morphologically transformed by HSV type 1 (HSV-1) or by the DNA from the transforming region of HSV-2 (the *Bgl*II N fragment). To demonstrate that this event is initiated by virus infection, we also report on the hypomethylation of host cell DNA synthesized during HSV-2 infection of primary rat embryo cells and provide evidence for the limited reincorporation of methylcytosine from degraded cellular DNA into viral DNA.

MATERIALS AND METHODS

Biological materials. Rat embryo control cells were prepared from 18-day-old embryos of the Hooded Lister rat (9). Rat embryo Asyn$^+$C cells are rat embryo cells transformed at nonpermissive temperature with the HSV-1 strain 17 temperature-sensitive, (*ts*) mutant *ts*A with morphology of syn$^+$. C designates the clone. Bn3 and Bn5 are two different clones of cells transformed after transfection of the *Bgl*II N fragment of HSV-2 strain HG52 which encodes the small subunit of ribonucleotide reductase activity and initiates morphological transformation (9). Bn5T is a tumor cell line derived after injection of Bn5 cells into inbred Hooded Lister rats. Cells were grown in BHKC13 medium supplemented with glutamine and 10% fetal calf serum (all from GIBCO Biocult, Paisley, U.K.). Wild-type HSV-2 strain HG52 (42) and *ts* mutants were grown and their titers were determined in BHKC13 cells. The *ts* mutants used in this study were *ts*K and *ts*1204 with a nonpermissive temperature of 38.5°C and *ts*1207 with a nonpermissive temperature of 39°C; all have a permissive temperature of 31°C. Cells were infected at 10 PFU/cell; wild-type virus was adsorbed at 37°C and *ts* virus was adsorbed at the nonpermissive temperature for 1 h; unadsorbed virus was removed by three washes in phosphate-buffered saline. At the nonpermissive temperature cells infected with the *ts*K mutant make the immediate early polypeptides Vmw 110, 65, 63, and 12 and a defective Vmw 175 in addition to the early polypeptide Vmw 136 (the large subunit of ribonucleotide reductase) (36). *ts*1204 has a defect in virus assembly (V. G. Preston, personal communication)

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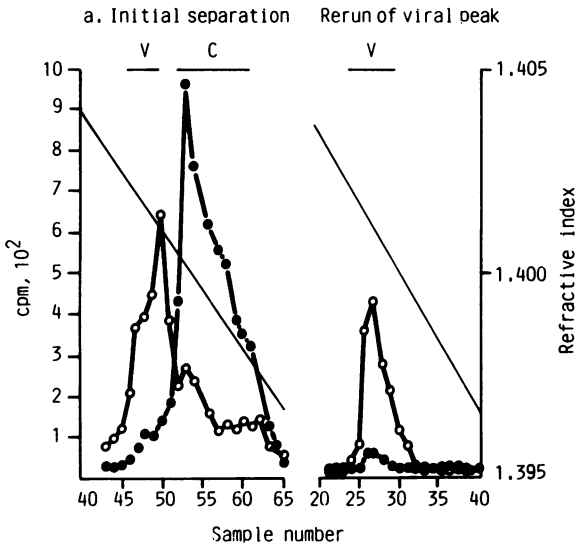

FIG. 1. Fractionation of cellular and viral DNA on gradients of CsCl. Results show the counts per minute in 5-μl samples taken across the gradient from cells prelabeled with [2-^{14}C]deoxycytidine (●) and labeled on infection with [6-^{3}H]uridine (○). The straight line shows the refractive index as measured on every fifth sample.

and *ts*1207 has a defect in the large subunit of ribonucleotide reductase activity (37). Both *ts*1204 and *ts*1207 were the kind gift of V. G. Preston.

Labeling. [6-^{3}H]uridine (Amersham International, Amersham, United Kingdom) was used at 50 μCi per 50-mm dish. Labeling of infected cells was carried out at 1 or 4 h postadsorption for a period of 4, 8, 16, or 24 h postinfection. Cells were prelabeled for 3 days with 50 μCi of [6-^{3}H]uridine or, in double-label experiments, 2.5 μCi of [2-^{14}C]deoxycytidine (New England Nuclear Corp., Boston, Mass.). Cycloheximide (Sigma, London, U.K.) was used at a final concentration of 50 μg/ml.

Separation of viral and host cell DNA. DNA was isolated from control and infected cells (9) and centrifuged to equilibrium on gradients of CsCl (initial refractive index, 1,400; speed, 33,000 rpm; time, 50 h). Figure 1a shows the results of an initial separation of viral and cellular DNA and Fig. 1b shows a second centrifugation of the viral DNA peak from cells labeled for 3 days with [2-^{14}C]deoxycytidine followed by the addition of [6-^{3}H]uridine at 4 h postinfection. Cells were harvested at 16 h.

Base analysis of DNA. Radiolabeled DNA was hydrolyzed with formic acid, and the bases were separated on Aminex A6 (Bio-Rad Laboratories, Richmond, Calif.) as described previously (4). The results are expressed as the percentage of cytosines methylated, i.e., counts per minute in methylcytosine × 100/counts in cytosine plus methylcytosine.

DNA methylase assays. DNA methylase was extracted from frozen cell pellets by lysis in hypotonic buffer containing 1% Tween 80, followed by shaking in 0.2 M NaCl for 20 min. Particulate matter was sedimented at 2,000 rpm for 10 min. Protein in the extract was estimated by the method of Bradford (7), and DNA methyltransferase was estimated as described previously (1).

TABLE 1. Proportion of cytosines (C) methylated in DNA of various HSV-2 transformed rat embryo cells[a]

Rat embryo cells	(mC × 100)/(C + mC) (no. of analyses)
Parent cell line	3.75 ± 0.07 (7)
Bn3	3.28 ± 0.41 (6)
Bn5	2.60 ± 0.37 (10)
ASynC^{+}	2.62 ± 0.28 (2)
Bn5T	1.70 ± 0.22 (7)

[a] Cells, growing in 90-mm plates, were labeled for 3 days with [6-^{3}H]uridine (50 μCi per plate), and the DNA was extracted and analyzed as described in Materials and Methods. The results present the mean ± standard deviation. mC, Methylcytosine.

RESULTS

We have looked at the level of DNA methylation in several lines of transformed cells and investigated whether the changes we see could be a direct result of herpesvirus infection. We considered that the virus may bring about a demethylation of preexisting cellular DNA or that it may interfere with the methylation of DNA synthesized postinfection.

Transformed cell lines. Table 1 shows the proportion of cytosines methylated in the DNA from a series of HSV-2-transformed rat embryo cells. By Southern blot analysis, these cells show no evidence of >500 base pairs of HSV-2 DNA present at 0.1 copy per cell but are transformed as judged by morphology, growth characteristics, and the ability to induce tumors in immunocompetent and immunodeficient animals (8). In addition, they have alterations in the expression of cellular polypeptides characteristic of transformed cells (27).

All of the transformed cell lines contain DNA, which is undermethylated compared with that of the embryonic cells from which they were derived (Table 1). The extreme case is that of the Bn5T line, which has less than half the methylation of the parental cells. As indicated in Materials and Methods, the Bn5T cell line was obtained from a tumor induced by transformation of rat embryo cells with the *Bgl*II N fragment of HSV-2 which encodes the small subunit of ribonucleotide reductase, although no enzymatic activity can be detected in the transformed cells (9).

We considered that such transformation could be brought about by the virus affecting the level of methylation of, and consequently the expression of, certain growth-controlling genes, and this paper addresses the question of whether HSV-2 infection can lead to a change in gross methylation status of the infected cell DNA.

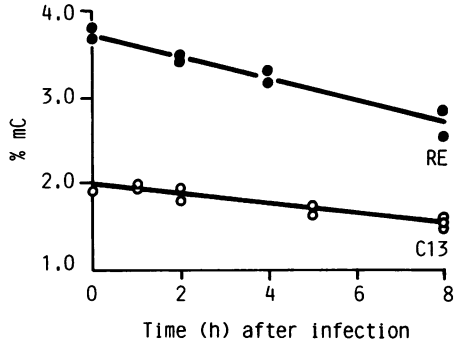

FIG. 2. Overall level of methylation of prelabeled DNA on infection with HSV-2. The counts in methylcytosine (mC) are expressed as a percentage of counts in cytosine plus methylcytosine. The cells (primary rat embryo [RE] or BHK C13 cells) were prelabeled with [6-^{3}H]uridine for 3 days prior to infection.

TABLE 2. Percentage of radioactive cytosines (C) methylated in cellular and viral DNA on infection of [2-^{14}C]deoxycytidine-prelabeled rat embryo cells with HSV-2[a]

Time after infection (h)	% mC [(mC × 100)/(C + mC)]		C/T ratio[b]
	Cellular	Viral	
0	3.70 ± 0.15 (9)		1.15 ± 0.02 (3)
4	3.64 ± 0.11 (2)	ND	1.25 ± 0.04 (3)
8	3.66 ± 0.21 (4)	0.61 ± 0.08 (2)	1.46 ± 0.07 (3)
16	3.69 ± 0.09 (7)	0.82 ± 0.40 (4)	1.03 ± 0.07 (3)
24	3.83 ± 0.14 (4)	1.13 ± 0.23 (2)	ND

[a] Rat embryo cells prelabeled for 3 days with [^{14}C]deoxycytidine (2.5 μCi per plate) were infected with HSV-2. [6-^{3}H]uridine was also added shortly after infection (see Table 3). DNA was purified on gradients of CsCl (Fig. 1) and analyzed as described in Materials and Methods. The results are the mean ± standard deviation with the number of estimations in parentheses. mC, Methylcytosine; ND, not determined.
[b] Ratio of [^{14}C]cytosine plus methylcytosine to [^{14}C]thymine in the total DNA.

Infection with wild-type virus. (i) Demethylation of preexisting DNA. Figure 2 shows that, when cells prelabeled for 3 days with [6-^{3}H]uridine or [2-^{14}C]deoxycytidine are infected with HSV-2 in the absence of further added radioisotopes, the level of methylation of the prelabeled DNA falls by 28% over an 8-h period. Further investigations in which the cellular and viral DNAs were separated in CsCl gradients prior to the analysis shows that this fall in the level of DNA methylation is not attributable to a demethylation of [2-^{14}C]deoxycytidine-labeled cellular DNA labeled prior to virus infection. There is no significant change on infection in the percent methylation of [^{14}C]cytosine in cellular DNA labeled prior to viral infection (Table 2). The results in Fig. 1 can therefore be largely attributed to the breakdown of some cellular DNA and the reincorporation of radioactivity into viral DNA. There is obviously a selection against reincorporation of methyl dCMP into the viral DNA as shown by the fact that, although on degradation the cellular DNA would yield a pool of dCMP in which 3.7% of the radioactive cytosine is methylated, reincorporation occurs in the proportion of methyl dCMP to total dCMP of about 0.7% up to 16 h postinfection. It is of interest, however, that the newly synthesised viral DNA does contain methylcytosine, though we cannot assess the total amount from this experiment as we do not know the proportion of viral DNA made from recycled nucleotides.

(ii) Methylation of DNA synthesized postinfection. When [6-^{3}H]uridine is added to rat embryo cells shortly after viral infection, the viral DNA synthesized contains very little, if any, radiolabeled methylcytosine (Table 3). The values are not significantly different from zero, but it is possible that even the small amount found (amounting to no more than 20 methylcytosines per HSV genome) results not from methylation of viral DNA but from methylation of newly synthesized cellular DNA followed by its breakdown and the reincorporation of the radioactive nucleotides into viral DNA. Not only is viral DNA essentially unmethylated, but it is very clear that, following viral infection, the level of methylation of newly synthesized cellular DNA is less than one-sixth of that found in mock-infected cells (Table 3). There is apparently a rapid inhibition of methylation of newly synthesized cellular DNA on infection, and this observation led us to investigate the activity of DNA methylase present in infected rat embryo cells.

(iii) Changes in DNA methylase activity. Following infection of cells with HSV-2 virus, there is a 70% fall in the specific activity of extractable DNA methylase by 16 h (Fig. 3). Mock-infected cells show a rise in activity over this time period. This rise probably represents the changes brought about by the stimulation of cell division by the change in medium. However, no significant change is seen in the 4 h postinfection, and in a repeat experiment the specific activity of DNA methylase extracted from nuclei prepared from cells at 0, 1, 2, 3, and 4 h postinfection was, respectively, 1.5, 1.4, 1.6, 1.6, and 1.4 U per mg of protein. From these data it is unlikely that a loss of DNA methylase is responsible for the 85% inhibition of cellular DNA methylation which occurs by this time. In general, tumor cells, although having lower levels of methylcytosine than the untransformed control cells, show either the same (5) or elevated (25) levels of DNA methyltransferase, and this is perhaps primarily a reflection of the rate of cell division. It is also unlikely that an imbalance in the supply of *S*-adenosylmethionine (5) is the cause of the inhibition of methylation.

Infection with mutant virus. We have also studied the effect on DNA methylation of infection of rat embryo cells, at the nonpermissive temperature, by certain *ts* mutants of HSV-2. Table 4 shows that infection with *ts*1204, which has a lesion in packaging, has the same effect as infection by wild-type virus, i.e., 10-fold reduction of methylation of newly synthesized DNA. However, infection with *ts*1207 (lesion in ribonucleotide reductase) or *ts*K (lesion in Vmw 175) has a much less dramatic effect on DNA methylation. Thus, there is no significant decrease in the percent methyl-

TABLE 3. Percentage of tritiated cytosines (C) methylated in cellular and viral DNA following infection of rat embryo cells[a]

Time after infection (h)	% mC [(mC × 100)/(C + mC)]		C/T ratio[b]
	Cellular	Viral	
16 (mock infection)	3.25 ± 0.10 (6)		0.80 ± 0.02 (3)
4	0.27 ± 0.07 (2)		6.61 ± 0.56 (3)
8	0.48 ± 0.04 (2)	0.08 ± 0.08 (2)	5.57 ± 0.52 (3)
16	0.42 ± 0.06 (7)	0.11 ± 0.07 (4)	3.40 ± 0.63 (4)
24		0.09 ± 0.04 (2)	ND

[a] Cells were labeled shortly after infection with [6-^{3}H]uridine as described in Table 2, footnote *a*. See Table 2 for abbreviations.
[b] Ratio of [^{3}H]cytosine plus methylcytosine to [^{3}H]thymine in the total DNA. There was no difference between viral and cellular DNA.

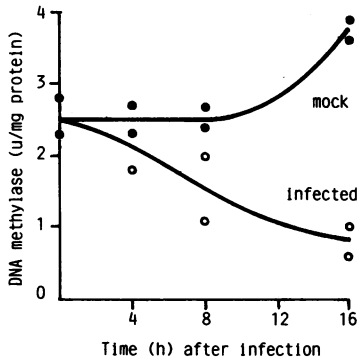

FIG. 3. DNA methyltransferase activity after infection of rat embryo cells with HSV-2. Enzyme activity was measured in cellular extracts as described in Materials and Methods. The results are for rat embryo cells infected with HSV-2 (○) or for a mock infection (●).

ation of total ^{14}C-prelabeled DNA and only a 20 to 25% decrease in methylation of total newly synthesized DNA. This implies that the inhibition of methylation is not solely dependent on the immediate early proteins synthesized by *ts*K mutants but probably requires production of the proteins (including ribonucleotide reductase) necessary for optimal synthesis of viral DNA. This is confirmed by infection with wild-type virus in the presence of cycloheximide when only Vmw 65, the virion polypeptide, is present.

Effect on radioactive C/T ratio. On infection of ^{14}C-prelabeled cells with wild-type HSV-2, the proportion of radioactivity recovered in DNA cytosine plus methylcytosine increases relative to that in DNA thymine: the C/T ratio (Table 2). This effect is not seen on infection with *ts*1207 or *ts*K at the nonpermissive temperature (Table 4: note the ratio differs from experiment to experiment; cf. Tables 2 and 4). The change in ^{14}C-prelabeled C/T ratio reflects an unequal reincorporation of [^{14}C]dCMP and [^{14}C]dTMP into DNA synthesized after infection, which may in part be a result of the higher G+C content of viral DNA. A less likely explanation is that there is a selective breakdown of T-rich DNA.

The former interpretation is borne out by study of the ^{3}H-labeled C/T ratio in DNA made from [6-^{3}H]uridine after infection (Tables 3 and 4). The change in ratio is very much more dramatic in this case, rising over 10-fold from about 0.8 in mock-infected cells to 8.7 in cells infected with wild-type virus. Taking this change to be a result of productive infection, it is clear that the *ts*1207 (ribonucleotide reductase) mutant does make low levels of virus DNA, but this does not occur with the *ts*K mutant or the wild-type virus in the presence of cycloheximide. We have shown that the elevation in the ratio of [^{3}H]cytosine plus methylcytosine to [^{3}H]thymine is typical of both newly synthesized cellular and viral DNA.

The explanation for the decreased incorporation of radioactivity into DNA thymine relative to DNA cytosine obviously cannot lie entirely in the higher G+C content of viral DNA, but it may lie in the increased pool sizes of dTTP found in infected cells. These pools are elevated as a result of the induction of the viral pyrimidine kinase which causes increased salvage of exogenous thymidine (20, 21), though it is not immediately clear what the source of such thymidine may be. The increased dTTP pool inhibits dCMP deaminase and ribonucleotide reductase, thereby reducing incorporation of [^{3}H]uridine into dTTP. Whether the small increase in the ^{3}H-labeled C/T ratio in the *ts*1207-infected cells reflects a low level of infection and only a small production of viral pyrimidine kinase or whether it illustrates a major involvement of viral ribonucleotide reductase in the pool size alterations has not been established. However, as the percent methylation of newly synthesized DNA is only slightly reduced, it is probable that there is a low level of virus replication in these cells.

DISCUSSION

Rat embryo cells show a very consistent level of DNA methylation, 3.7%, as measured by the ratio of radioactive methylcytosine to total cytosine following a 3-day incubation with either [6-^{3}H]uridine or [2-^{14}C]deoxycytidine. However, when labeled for only 4, 8, or 16 h (Table 3), the measured proportion of newly incorporated cytosine methylated is significantly lower, 3.2%. This confirms previous observations (3, 10, 43) and reflects the delay between synthesis of DNA and its complete methylation, a process which may take several days in a population of cells approaching a stationary phase of growth in which DNA methylase may be limiting (2, 10).

HSV-1 DNA has been reported to be transiently methylated during the lytic cycle (40) and Epstein-Barr virion DNA is highly methylated (11). In transformed cells, Epstein-Barr viral DNA becomes increasingly methylated with time (23), but on induction with phorbol ester and *n*-butyrate one highly methylated Epstein-Barr virus producer line was dramatically demethylated in the host and viral DNA prior to Epstein-Barr virus amplification (41). Other less methylated lines did not show this demethylation on induction. This demethylation event, which is similar to one that occurs in mouse erythroleukemia cells on induction of differentiation (38), has been postulated to occur by the gross removal of methylcytosine from the DNA and its replacement with cytosine. The present data rule out the occurrence of a

TABLE 4. Percentage of radioactive cytosines (C) methylated and radioactive C/T ratio of ^{14}C-prelabeled DNA and ^{3}H-labeled DNA synthesized after infection with a series of viral mutants[a]

Mutant or treatment	% mC [(mC × 100)/(C + mC)]		C/T ratio (%)[b]	
	^{14}C prelabeled	^{3}H labeled (2–8 h)	^{14}C prelabeled	^{3}H labeled (2–8 h)
Mock infected	4.01 ± 0.30	3.34 ± 0.16	0.56 ± 0.06	0.78 ± 0 (3)
Wild type	2.83 ± 0.17	0.17 ± 0.05	0.78 ± 0.05	8.72 ± 0.71 (3)
Wild type + cycloheximide	3.57 ± 0.53	2.20 ± 1.73	0.69 ± 0.12	0.58 ± 0.11 (3)
*ts*1204	3.25 ± 0.38	0.37 ± 0.09	0.73 ± 0.16	7.13 ± 1.93 (3)
*ts*1207	3.88 ± 0.30	2.48 ± 0.21	0.52 ± 0.10	1.37 ± 0.09 (3)
*ts*K	3.99 ± 0.79	2.60 ± 0.69	0.59 ± 0.10	0.84 ± 0.16 (5)

[a] The cells were grown and labeled as described in Tables 2 and 3, footnote *a*. Cellular and viral DNA are not analyzed separately. The numbers of estimates are given in parentheses.

[b] See Tables 2 and 3, footnote *b*.

demethylation event taking place by this mechanism as prelabeled cellular DNA does not preferentially lose methyl groups or methylcytosine following infection with HSV-2 (Table 2). Furthermore, the C/T ratio in [^{14}C]deoxycytidine-prelabeled rat embryo cellular DNA does not fall on infection with HSV-2, showing that there is no selective replacement of preexisting cytosine plus methylcytosine with newly synthesized, unlabeled material as we have shown occurs in erthroleukemia cells (Adams and Rinaldi, unpublished observations). Indeed, the reverse is true (Table 2), which may be partly attributed to a breakdown of relatively A+T-rich cellular DNA and synthesized of G+C-rich virus DNA up to 8 h postinfection.

The overall loss of prelabeled methylcytosine following infection apparent in Fig. 1 is a result of the degradation of some radiolabeled cellular DNA followed by its incorporation into newly synthesized viral DNA. Such a reincorporation event was observed many years ago (P. A. J. Perera, Ph.D. thesis, University of Glasgow, Glasgow, U.K., 1970) and has recently been reinvestigated by Nutter et al. (32). We observe that by 8 h postinfection about 11% of the prelabeled cellular DNA has been degraded and the nucleotides released have been reincorporated into viral DNA. The reincorporation event is selective to some extent in that the reincorporation of cytosine over methylcytosine is preferred. This presumably reflects the specificity of the viral DNA polymerase or kinases or both.

We should point out that this methylcytosine in viral DNA is not formed postsynthetically as a result of methyltransferase activity, but results from incorporation of preformed methyl dCMP. As such it would be expected to replace cytosine at random and would therefore not occur exclusively in the mCG dinucleotide. These methylcytosines incorporated into viral DNA may interfere with the expression of viral genes, and this suggests to us a possible therapeutic use of methyldeoxycytidine on the treatment of herpesvirus infections.

There is a limited amount of cellular DNA synthesized following HSV infection of rat embryo cells. The cellular DNA made in the 16 h following infection shows a considerably reduced level of methylation (0.4% relative to 3.2%), and this is also true for DNA made as early as 4 h postinfection (Table 3).

In certain cases, in particular with the cells infected in the presence of cycloheximide, the recovery of cells and hence radioactivity is particularly poor, and this explains the high coefficients of variation obtained in some cases. This is, of course, accentuated when the percent methylation is reduced. This in no way detracts from the significance of the observation that methylation of cellular DNA is inhibited on viral infection, an observation which would occur by chance very much less than once in 1,000 observations, i.e., $P \ll 0.001$.

The failure to methylate viral and cellular DNA following viral infection shows a positive correlation with the increased ^{3}H-labeled C/T ratio in the newly synthesized DNA. Both probably are dependent on the production of some early viral proteins essential for viral replication.

We are currently investigating the effect of a battery of HSV mutants, each carrying a specific lesion, to determine the mechanism by which DNA methylation is inhibited post-HSV infection.

Conclusion. Our results show a massive inhibition of methylation of DNA synthesized following infection of rat embryo cells with HSV-2. Although we found no evidence for demethylation of previously synthesized cellular DNA, the evidence from the transformed cell lines is consistent with the persistence in such cells of hypomethylated DNA produced shortly after an abortive infection. Although no viral DNA remains in these transformed cells, the resulting undermethylation of cellular DNA is postulated to have activated certain cellular genes.

ACKNOWLEDGMENTS

Thanks are due to R. M. S. Smellie and J. H. Subak-Sharpe for the provision of facilities and to the Medical Research Council for financial support to R.L.P.A.

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