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Methylation of HSV-1 DNA as a mechanism of viral inhibition: studies of an analogue of methyldeoxycytidine: trifluoromethyldeoxycytidine (F_3mdCyd)

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Summary

Although several hypomethylating agents such as 5-azadeoxycytidine and 5-fluorodeoxycytidine have been shown to activate transcription after incorporation into viral or cellular DNA, agents which selectively affect the methylation status of virus-infected cells have not been described.

Studies on the antiviral effect of the methyldeoxycytidine (mdCyd) analogue trifluoromethyldeoxycytidine (F_3mdCyd) showed significant antiviral activity against herpes simplex virus type 1 (HSV-1). This analogue of both dCyd and dThd is selectively incorporated into the DNA of herpesvirus infected cells due to the unique specificity of the herpesvirus thymidine kinase (TK) because the HSV-1 TK is both a dCyd and dThd kinase. In contrast, the deoxycytidine kinase of uninfected cells preferentially phosphorylates dCyd and has a poor affinity for F_3mdCyd.

F_3mdCyd hemisubstituted M13 DNA displayed the same properties as mdCyd-substituted M13 DNA with respect to cleavage by restriction enzymes, and acted as an efficient template for eukaryotic DNA methyltransferase (*S*-adenosyl-L-methionine DNA (cytosine-5) methyltransferase: EC 2.1.1.37). Using the persistently infected CEM cell model system, the extent of DNA methylation was shown to increase in a dose-related manner when HSV-1-infected CEM cells were treated with increasing concentrations of F_3mdCyd. Higher levels of methylation correlated with significant decreases in HSV-1

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titers. Isoschizomer analyses followed by Southern blotting and hybridization with genomic HSV-1 DNA showed that DNA from HSV-1-infected, analogue-treated Vero cells was resistant to cleavage by restriction enzymes at a time when productive virus was not present in culture. We infer from these results that the methylation-like properties of the incorporated F_3mdCyd occur concomitantly with, and appear to be involved in, the mechanisms of the analogue's antiviral effect towards HSV-1.

Methylation; HSV-1; Trifluoromethyldeoxycytidine

Introduction

Direct correlations of hypomethylation with transcriptional activity and hypermethylation with transcriptional inactivity in viral systems have been repeatedly demonstrated. Strong associations have been shown between the absence of transcriptional or transforming activity and the presence of the methylated state of DNA in several diverse viral systems which include adenoviruses and herpesviruses (Vardimon et al., 1980; Sutter and Doerfler, 1980; Desrosiers et al., 1984; Hoffman and Diala, 1983; Saenudsen et al., 1983). Transcriptional inactivation due to hypermethylation has been demonstrated for the retroviruses MMTV, avian sarcoma virus, Moloney murine leukemia virus and HIV-1 (Varmus et al., 1973; Cohen, 1980; Guntaka et al., 1980; McGeady et al., 1983; Searle et al., 1984; Bednarik et al., 1987,1991). Hypermethylating agents (such as DNA analogues which may act as templates for DNA methyltransferase) may have the potential to silence transcription as well as interfere with other viral replication processes. The use of such agents, which are specifically incorporated into the DNA of herpes virus infected cells but not in uninfected cells, to affect latency and recurrent infections as well as interfere with virus replication, was first suggested in this laboratory (Fox et al., 1983).

In general, DNA isolated from intact viruses (polyoma virus, SV40 virus, adenovirus, HSV-1, Epstein-Barr virus, mouse mammary tumor virus, Moloney murine leukemia virus, Baculovirus), and free (non-integrated) intracellular viral DNA in productively and abortively infected cells, is not detectably methylated (Kaye and Winocour, 1967; Fiers et al., 1978: Gunthert et al., 1976; Whitby et al., 1987; Low et al., 1969; Sharma and Biswal, 1977; Varmus et al., 1973; Stuhlman et al., 1981; Tija et al., 1979). The highly efficient and rapid rate of viral DNA replication in productively infected cells, as well as the close association of DNA methyltransferase with cellular DNA (Burdon, 1985) may be some of the factors which account for the lack of virion DNA methylation. Yet when viral DNA is integrated into a transformed cell, some (if not all) of the viral DNA becomes methylated (Vardimon et al., 1980; Doerfler et al., 1982).

As with eukaryotic systems, exceptions in viral systems have occasionally been noted in which conditions of hypomethylation are not accompanied by genetic expression (Kuhlman et al., 1982); however, it is important to emphasize that in viral systems (unlike the case with eukaryotic systems) hypermethylation *with* genetic expression has not yet been conclusively established. This observation categorically infers that correlations between *hyper*methylation states and genetic down-regulation may have far more importance in viral systems.

F_3mdCyd possesses a low K_m with respect to the herpesvirus pyrimidine nucleoside kinase, and a very high K_m with respect to the separate mammalian deoxycytidine and thymidine kinases (Dobersen and Greer, 1978). In both in vitro and in vivo studies from this laboratory, 5-substituted analogues of deoxycytidine have been shown to possess significant antiviral activity towards herpesviruses even when deamination is prevented (Greer et al., 1975; Fox et al., 1979,1982,1983). However, the molecular mechanisms of the HSV-1 antiviral effects of these 5-substituted analogues of deoxycytidine were not investigated prior to the present study.

In studies using (*E*)-5-(2-bromovinyl)-2′-deoxycytidine against HSV-1, singly and in combination with deaminase inhibitors (H_4Urd, H_2dUrd, and H_4dUrd) DeClercq and co-workers (Aduma et al., 1990a) support the findings that halogenated analogues of dCyd are more selective inhibitors of HSV-1 than corresponding analogues of deoxyuridine (which do not affect the methylation status of DNA) (Singer et al., 1977; Kaysen et al., 1986).

Recent studies have suggested that agents such as 5-bromodeoxycytidine (BrdCyd) and 5-iododeoxycytidine (IdCyd), have the capacity to act as a substrate for DNA methyltransferase after their incorporation into DNA (Hardy et al., 1988). This effect is hypothesized to occur because the Van der Waals radius of the bromine (1.95 Å) and iodine (2.15 Å) atom approaches 2 angstroms, which is the dimension of the CH_3 group in the 5-position of the pyrimidine. The major function in vivo of DNA methylases is the maintenance of the pattern of methylation. The natural substrate of DNA methyltransferase is the hemimethylated DNA that results following DNA replication and in vitro, the enzyme shows much greater activity with hemimethylated DNA than with native or denatured DNA (Adams et al., 1979; Jones and Taylor, 1981; Taylor and Jones, 1982). Because of the similarities to the methyl-moiety, Hardy et al. (1988), proposed that the methyltransferase enzyme will recognize bromo or iodo 5-substituted analogues as 5-methyldeoxycytidine, and proceed to methylate the newly synthesized DNA strand as directed by the incorporated analogue. The purpose of these studies was to determine if the same reaction mechanisms occur after F_3mdCyd is incorporated into DNA. The CF_3 moiety at the 5-carbon position of F_3mdCyd possesses a Van der Waals radius of 2.4 Å, and thereby relates our findings and those of Hardy et al. (1988) to previous studies in our laboratory in which we have shown that IdCyd and BrdCyd possess substantial anti-HSV-1 and 2 activity when deamination is prevented (Fox et al., 1983). F_3mdCyd (with or without H_4Urd) and F_3dThd have been

previously shown in this laboratory to result in greater than four logs of inactivation of HSV-1 and HSV-2 in HEp-2 cells at a concentration of 0.03 mM. F_3mdCyd without H_4Urd and F_3dThd resulted in 0.02 and <0.002% survival, respectively, whereas F_3mdCyd with H_4Urd resulted in 80% survival of HEp-2 cells at a concentration of 0.03 mM, as determined in a clonogenic assay (Fox, 1979). Thus, F_3mdCyd has a great potential for selectivity when it is coadministered with H_4Urd.

The goal of these studies was to determine whether methylation of DNA could be a component of the antiviral effect of these analogues. Specifically, we wanted to determine the effects of F_3mdCyd in: (1) serving as a methylation signal to methyl-sensitive restriction enzymes after hemisubstitution into M13 DNA constructs; and (2) serving as a template for the CEM host cell (or viral) methyltransferase after incorporation into the DNA of persistently infected CEM cells. Finally, we used restriction enzyme analyses and Southern blot procedures to determine that F_3mdCyd was incorporated into HSV-1 DNA and displayed methylation-like effects in DNA after treating infected Vero cells with the analogue.

Materials and Methods

Cells and cell culture

The CCRF-CEM (ATCC: CCL 119) and Vero (ATCC: CCl 81) cells were obtained from the American Type Culture Collection (Rockville, MD). CEM cells and Vero cells were cultured in RPMI medium and Eagle's medium, respectively, supplemented with 10% fetal calf serum containing 2 mM glutamine and antibiotics (penicillin and streptomycin).

Virus strains and propagation

Herpes simplex type 1 (strain F) was used. All virus stocks were grown and titrated on Vero cells and stored in small aliquots at $-70°C$.

Nucleosides, nucleotides, and their analogues

Pyrimidine bases, nucleosides and nucleotides were purchased from Sigma Chemical Company. Deoxytetrahydrouridine (H_4dUrd) was supplied at >99% purity by Calbiochem-Behring, Inc. It was important that it was free of contamination with deoxyuridine and dihydrodeoxyuridine in view of their antagonism of the antiviral effect of another deoxycytidine analogue, 5-methoxymethyl-2′-deoxycytidine (Aduma et al., 1990). F_3mdCyd was supplied by Dr. Gene Stump of PCR, Inc., Gainesville, FL.

Radiochemicals

[6-^{3}H]dCyd (specific activity, 14 Ci/mmol) and [*methyl*-^{3}H]*S*-adenosylmethionine (specific activity, 11.8 Ci/mmol) were purchased from New England Nuclear. [^{3}H]F_3mdCyd (final specific activity, 11.8 mCi/mmol) was obtained by custom tritiation from New England Nuclear.

CEM infection by HSV-1

Log phase CEM cells were infected with HSV-1 as previously described by Hammer et al. (1980). Briefly, 1.2×10^7 cells were incubated with HSV-1 in a volume of 1 ml (multiplicity of infection of 1 plaque forming unit (PFU)/cell) for 1 h on a shaker apparatus at 37°C. Cells were subsequently washed 3 times in medium, resuspended to a concentration of $4–5 \times 10^5$ cells/ml and incubated at 37°C. At 2–3-day intervals, cells were passaged by adding fresh medium to the cell suspension to achieve a final concentration of $4–5 \times 10^5$ cells/ml. Only persistently infected CEM cells, which were maintained in culture for 3 months or longer and showed production of infectious virus, were used for studies with F_3mdCyd. CEM cell suspensions were assayed for the presence of infectious virus as previously described by Schildkraut et al. (1975) on a biweekly basis.

For FACS analyses, 1×10^6 persistently infected CEM cells were treated with a 1:100 dilution of rabbit polyclonal anti-HSV-1 antibody labeled with FITC (Accurate Chemicals, Westbury, NY) in a direct labeling procedure for 1 h at 4°C. Cells were then washed and resuspended to 1 ml in cold PBS. Antibody-treated cells were analyzed using the Coulter Electronics (Epics 731) Model, tuned to 488 nm with filter set-up for dual light scatter and fluorescein emission at 525 nm.

Treatment of cell cultures with pyrimidine analogues

Log phase CEM or Vero cells were standardized to a density of 1×10^5 cells/ml and treated with various analogue concentrations for 72 h at 37°C before harvest for HPLC, plaque assay or Southern blot analyses. In some studies, after 72 h of analogue treatment, CEM cells were washed 2× with PBS, utilizing 3 low-speed centrifugations, and media was replaced in cultures either with or without analogue. CEM cells were then harvested for HPLC, plaque assay or Southern blot analyses.

Cell DNA harvest and purification for HPLC or restriction digestion

Log phase cells were exposed to various drug treatment regimens using 3.75, 5.62, 7.50, 11.25, and 15.00 μM F_3mdCyd with 20 μM H_4dUrd (as described in text). After completion of the incubation at 37°C, cell DNA was harvested and purified for HPLC analyses as previously described by Briggle et al. (1986). Briefly, DNA samples were digested with 0.5 μg/ml ribonuclease A for 12 h at

37°C. After extraction with chloroform and determination of DNA concentration, samples were sheared several times via passage through a 26-gauge needle and digested with 300 U of DNAse I for 24 h at 37°C. The pH was adjusted to 8.5 and the samples were digested with 0.05 U of snake venom phosphodiesterase and 500 μg of bacterial alkaline phosphatase for 24 h at 37°C. DNA was loaded onto the column in 300-ng amounts. The HPLC column used was a Bondapak C_{18} (Waters Assoc.). The mobile phase consisted of 0.005 M tetrabutylammonium phosphate, 0.0025 M dibasic ammonium phosphate (pH 6.8) in 20% methanol. The flow rate was 0.7 ml/min with a detector sensitivity of 0.002 absorbance units full scale. Quantitation, sensitivity and reproducibility of the assay method have previously been established by Cieri (1980) and Briggle et al. (1986). Comparisons of estimates of analogue incorporation into DNA were performed using peak height area vs. isotopic labeling of analogue. Although comparable, isotopic labeling methods were selected as the most accurate method to determine the extent of analogue incorporation into DNA.

Restriction endonuclease digestion, agarose gel electrophoresis and Southern transfer

Total DNA from Vero cells was digested with restriction enzymes under conditions specified by the supplier, electrophoresed in a 1.5% agarose gel and transferred to Gene Screen Plus (Dupont). 200 μg denatured salmon sperm DNA and $1 \times 10^{8-9}$ cpm/μg ^{32}P-labeled genomic HSV-1 DNA probe (a gift from Vincent Vann, University of Miami School of Medicine, FL) labeled by random primer extension (Prime-A-Gene, Promega) was added to the hybridization mixture. Lambda DNA was added as an internal control to verify completeness of restriction digestion. The filters were washed 2 times in 5× SSPE, 0.5% SDS for 15 min at room temperature, 2 times in 1 × SSPE, 0.5% SDS for 15 min at 37°C, and 1 × with 0.1 SSPE, 1.0% SDS for 15 min at 37°C. Partially dried nylon membranes were sealed in Saran Wrap, exposed to Kodak XAR X-ray film for various times at room temperature, with two Dupont Cronex intensifying screens.

Construction of analogue hemisubstituted M13 DNA

Hemisubstituted DNA was synthesized in vitro by primer extension of single-stranded M13 DNA as template. The reaction mixture (15 μl) contained 20 μM each of dATP, dTTP, dGTP, 10 μCi (15 μM) [^{32}P]dGTP, (as a non-competitive isotopic label with respect to dCTP) and 20 μM dCTP, 5-mdCTP, or F_3mdCTP (obtained from Sierra Bioresearch, Inc., Tucson, AZ), 66 mM Tris-HCl (pH 7.5), 6.7 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 μg of template single-stranded M13 DNA annealed to 0.8 pmol M13 primer, and 1 U of Klenow DNA polymerase I. The complementary strand was synthesized at 15°C for 24 h in the presence of T4 DNA ligase (2 Weiss units). After

incubation, 10 μg of unmethylated lambda DNA was added to the reaction mix as an internal control for restriction enzyme digestion. DNA was precipitated with ethanol and digested with the appropriate restriction enzyme. Under these conditions only lambda DNA was visible on the EtBr (ethidium bromide) stained gels, whereas only ^{32}P-labeled (hemisubstituted M13 DNA) was visible on the autoradiographs of these gels. Gels were stained with EtBr to view digestion of lambda DNA, then dried and exposed to film to view digestion of ^{32}P-labeled M13 DNA.

Extraction of CEM nuclear methyltransferase

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 2000 rpm for 10 min (Macnab et al., 1988). Protein in the extract was estimated by the method of Bradford (1976). DNA methylase activity of CEM nuclear fractions was determined by cpm incorporated into M13 DNA (hemisubtituted with mdCyd) derived from [*methyl*-^{3}H]SAM which was added to the reaction mix (see 'DNA methylation in vitro').

DNA methylation in vitro

Hemisubstituted DNA was used as a substrate for the CEM methyltransferase enzyme. In a final volume of 80 μl, 0.25 μg analogue hemisubstituted or 0.12 μg dCyd substituted template DNA, which served as a negative control, was incubated with 2 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (3 μCi/assay), 10 μg protein of partially purified CEM nuclear lysate in a buffer containing 10 mM Tris-HCl (pH 7.4), 30 mM NaCl, 10 mM EDTA, 0.2 mM DTT, 15% (v/v) glycerol for 2 h at 37°C. After incubation, reactions were terminated by addition of SDS to 0.6%, followed by a 20-min incubation at 37°C with 400 μg/ml Proteinase K. Two volumes of 0.5 M NaOH were then added and incubation continued for 10 min at 60°C. After cooling on ice, carrier salmon sperm DNA (20 μg/assay) was added to the mix and DNA precipitated by adding ice-cold TCA to 5%. Samples were washed extensively with 5% TCA. The precipitated DNA was then hydrolyzed at 100°C for 30 min. Samples were centrifuged and supernatants counted in scintillation fluid (Ecolume), in duplicate (Gruenbaum et al., 1982; Wilson and Jones, 1983).

Results

Effects of F_3mdCyd and F_3dThd on methylation-sensitive enzymes using M13 constructs

If the hypothesis that F_3mdCyd acts as a legitimate methylation signal to

methylation sensitive enzymes is correct, its molecular effects should parallel those of the positive (mdCyd) control. To test this hypothesis, experiments were performed to determine the effects of F_3mdCyd substitution in DNA on the activity of methyl-sensitive restriction enzymes.

M13 was used to study the effects of F_3mdCyd on *Hpa*II and *Msp*I cleavage. For details of hemisubstituted M13 construction, see Materials and Methods.

The EtBr stained gels in Figs. 1A and 2A show that both restriction enzymes *Hpa*II and *Msp*I completely cleave unmethylated lambda DNA in all cases, as expected, as well as replicative form (double-stranded) control M13 DNA (see figure legends for locations of lambda and M13 DNA in gels).

The autoradiographic exposure of these gels, however, display different digestion patterns for both F_3mdCTP and mdCTP, vs. dCTP hemisubstituted M13 DNA, in the absence of competitive dCTP during primer synthesis of M13 DNA. MdCTP and F_3mdCTP hemisubstituted M13 DNA was totally refractory to digestion by *Hpa*II (Fig. 1B; lanes 6,8) and *Msp*I (Fig. 1B; lanes 7,9) in the absence of competitive dCTP during primer synthesis of M13 DNA.

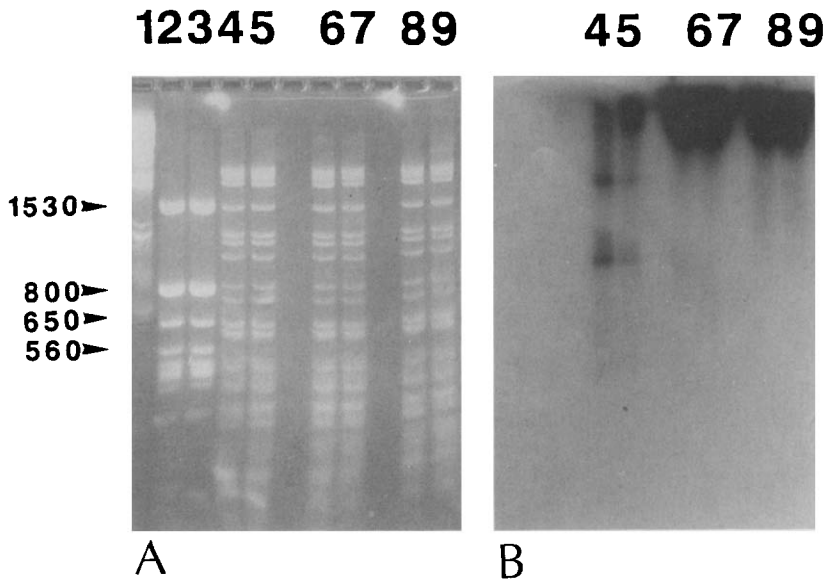

Fig. 1. Restriction digestion of hemisubstituted M13 DNA which is substituted by mdCyd, or F_3mdCyd at all dCyd sites. 10 μg of an unmethylated lambda DNA preparation and 0.25 μg of ^{32}P-labeled analogue hemisubstituted M13 DNA were digested by 18 U *Hpa*II and 20 U *Msp*I at 37°C for 18 h. Approx. 100 000 cpm of each ^{32}P-labeled reaction mixture was electrophoresed in a 1.5% agarose gel. The completeness of digestion of ^{32}P-labeled M13 DNA shown in **B** (autoradiographic exposure) was verified by the addition of unmethylated, unlabeled lambda DNA to all samples except for control replicative M13 DNA (lanes 2,3) and is shown in **A** (ethidium bromide stain). Lane 1: 1 μg lambda DNA digested with *Bst*I; lanes 2,3: unmethylated replicative form M13 DNA digested with *Hpa*II and *Msp*I, respectively; lanes 4,5: ^{32}P-labeled dC hemisubstituted M13 DNA digested with *Hpa*II and *Msp*I, respectively; lanes 6,7: ^{32}P-labeled mdCyd hemisubstituted M13 DNA digested with *Hpa*II and *Msp*I, respectively; lanes 8,9: ^{32}P-labeled F_3mdCyd hemisubstituted M13 DNA digested with *Hpa*II and *Msp*I, respectively.

These results reflect the absolute replacement of mdCTP or F_3mdCTP at all dCyd sites in M13 DNA, since no competitive dCTP was present in the reaction mix during the synthesis of double-stranded M13 by primer extension. Although described as 'methyl-insensitive', *Msp*I is partially or completely inhibited from digestion in these experiments when the substitution of F_3mdCyd or mdCyd occurs at the outer, or both cytosines, of the recognition sequence (Keshet and Cedar, 1983). Ten times the concentration of competitive dCTP (20 μM dCTP vs. 2 μM mdCTP or F_3mdCTP) was selected as a suitable ratio of excess dCTP vs. mdCTP or F_3mdCTP to display the competitive effects of dCTP vs. analogue incorporation into M13 DNA. During primer extension, (under these conditions), there was extensive digestion by both *Hpa*II and *Msp*I (Fig. 2B; lanes 6,8) and *Msp*I (Fig. 2B; lanes 7,9) of the mdCyd or F_3mdCyd hemisubstituted M13 construct. As predicted from the studies of Hardy et al. (1988), similar results were obtained using mCTP, IdCTP and BrdCTP.

Effects of partially or completely hemisubstituted M13 DNA on substrate specificity of CEM DNA methyltransferase

Experiments were performed to determine if DNA containing F_3methyldCyd acts as a template for the addition of ^{3}H-methyl groups from [*methyl*-^{3}H]*S*-adenosyl-methionine ([*methyl*-^{3}H]SAM) by DNA methyltransferase. F_3mdCTP or mdCTP (used as the positive control) were added under conditions of no competition with dCTP, or at equimolar concentrations (25 μM dCTP plus 25

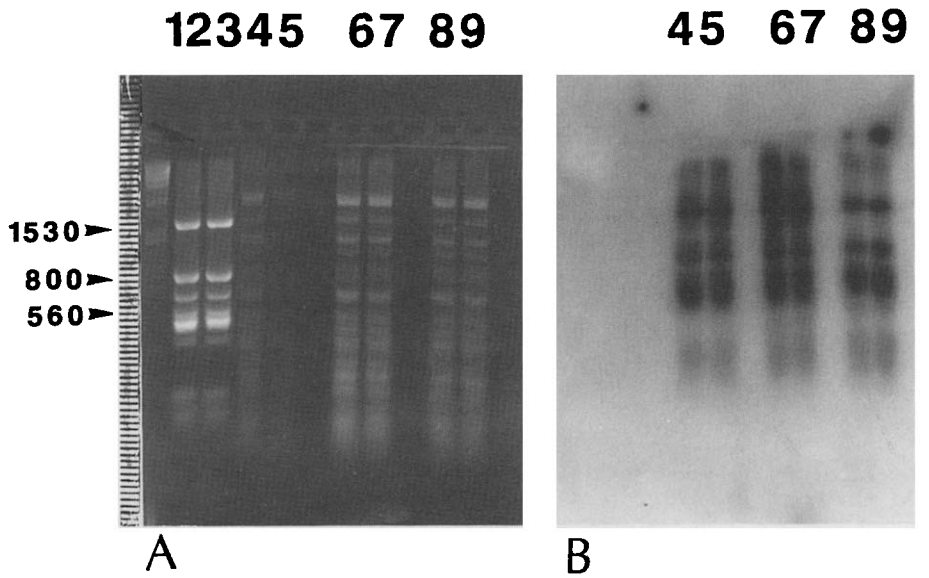

Fig. 2. Restriction digestion of hemisubstituted M13 DNA which is substituted by mdCyd, or F_3mdCyd at few dCyd sites. See legend to Fig. 1 for details of gel loading.

μM mdCTP or F_3mdCTP) during the initial M13 synthesis reaction. These completely (or partially) hemisubstituted M13 DNA preparations were then used as substrates for partially purified CEM nuclear extracts containing 10 μg protein, in the presence of [*methyl*-^{3}H]SAM. Maximal levels of methyl group addition (1060–1293 cpm/0.25 μg DNA) are seen when using fully substituted M13 constructs (Fig. 3). Equimolar levels of dCTP during primer extension effectively competed with analogue substitution of M13 DNA and relatively decreased methyltransferase activity to 487–501 cpm/0.25 μg DNA. That is, cpm incorporated into partially substituted M13 constructs (derived from [*methyl*-^{3}H]SAM) were reduced to 38–47% that of the completely substituted M13 construct (Fig. 3).

Selectivity of F_3mdCyd incorporation into the DNA of HSV-1-infected CEM cells

The CEM model system established by Hammer et al. (1981) will sustain a persistent herpesvirus infection for periods of 1 year or longer in continuous culture, with 95–100% viability. HSV-1 persistently infected CEM cell cultures consistently maintained a viral log titer which ranged between 3.3–3.7 $\log_{10}$

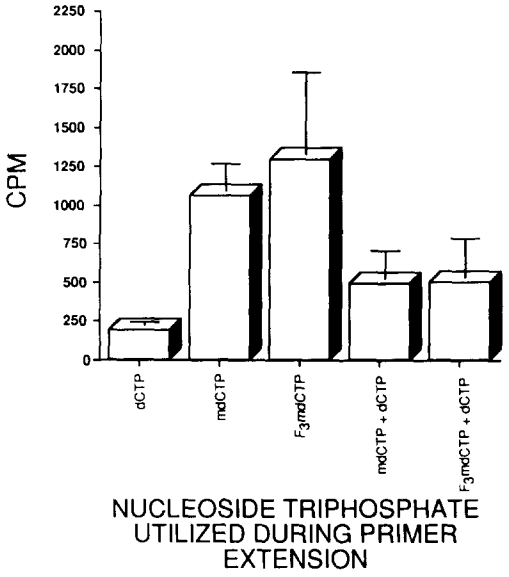

Fig. 3. Effects of partially or completely analogue hemisubstituted M13 DNA on substrate specificity of CEM methyltransferase. 0.25 μg of dCTP, mdCTP, or F_3mdCTP hemisubstituted M13 DNA was subjected to methylation by a partially purified extract of CEM methyltransferase enzyme in the presence of [*methyl*-^{3}H]*S*-adenosylmethionine at 37°C for 1 h. Equimolar concentrations of dCTP vs. mdCTP or F_3mdCTP were used during primer extension where indicated (mdCTP + dCTP or F_3mdCTP + dCTP) to observe dosimetric effects of CEM methyltransferase towards the partially hemisubstituted M13 substrate.

Values represent the mean cpm + SE of two separate determinations, performed in duplicate.

PFU/ml (data not shown). Other studies have shown that HSV-1 titers in persistent CEM cell cultures range between 4–6 $\log_{10}$ PFU/ml from 30–90 days after the initial HSV-1 infection (Rinaldo, 1979).

Fluorescent staining by FACS analysis using polyclonal anti-HSV-1 antibody showed that the number of CEM cells expressing HSV-1 antigens ranged from 31.2–36.8% of the total cell population three months after initial HSV-1 infection (data not shown). Other studies have shown that a maximum level of 40% of the CEM cell population express HSV-1 antigens in persistently infected cultures (Rinaldo et al., 1979). We used this cell system to determine methylation levels of F_3mdCyd-treated cultures during periods of inhibition of HSV-1 replication, as well as during periods of HSV-1 replication after removal of the analogue from cultures.

Experiments were performed to determine if F_3mdCyd would be selectively incorporated into HSV-1-infected CEM cells, and not uninfected CEM cells (Table 1). It was additionally important to determine if the deaminated product of F_3mdCyd (F_3dThd) would also be incorporated into the DNA of HSV-1-infected cells. It is unlikely that F_3dThd, which is a thymidine analogue, could act as a methylation signal for DNA methyltransferase. Additionally, if F_3mdCyd is converted to F_3dThd, less F_3mdCyd would be available for incorporation into DNA. Deoxytetrahydrouridine (H_4dUrd) was added as a (deoxycytidine and deoxycytidine monophosphate) deaminase inhibitor in these experiments (Maley and Maley, 1971).

Radioisotopically labeled [^{3}H]F_3mdCyd) was used to analyze analogue incorporation into HSV-1-infected CEM DNA. The material (which was obtained by custom tritiation) possessed a relatively low specific activity of 11.8

TABLE 1

Metabolites derived from labeled nucleosides in DNA from uninfected and HSV-1 infected CEM cells

Treatment μM F_3mdCyd	dCyd (pm)[c] specific activity	F_3mdCyd (pm) specific activity[a]	%dCyd substitution[a]
Uninfected/7.50	15.1 ± 0.3	$\leqslant$0.84[b]	$\leqslant$5.6[b]
Infected/0	28.1 ± 0.1	$\leqslant$0.84[b]	$\leqslant$2.9[b]
Infected/3.75	19.2 ± 0.7	1.99 ± 0.4	10.4 ± 2.1
Infected/7.50	14.3 ± 0.2	3.38 ± 0.9	23.6 ± 6.3
Infected/15.00	8.7 ± 0.1	2.48 ± 0.3	28.5 ± 3.4

[a]Picomoles (pm) F_3mdCyd incorporated into CEM DNA, as well as % dCyd substitution by F_3mdCyd, was calculated from specific activity of incorporated [^{3}H]F_3mdCyd (specific activity of [^{3}H]F_3mdCyd = 11.8 mCi/mmol).

[b]Minimum detectable background.

[c]Picomoles (pm) dCyd incorporated into CEM DNA was calculated from specific activity of newly incorporated [^{3}H]dCyd (specific activity of [^{3}H]dCyd = 14 Ci/mmol). Values represent the mean number of pmol incorporated + SE of 2 determinations.

mCi/mmol. For these procedures, three-minute sample fractions were collected and counted for radioactivity. When analyzing standardized (3 μg) DNA samples from HSV-1-infected, analogue-treated CEM cell cultures, we were able to show that radioactivity (713–2352 cpm) could be repeatedly recovered in HPLC fractions specific for F_3mdCyd (fractions 20–22). In these samples, radioactivity of 26–112 cpm, recovered as F_3dThd (fractions 34–36), was only observed when treating HSV-1-infected, analogue-treated CEM cell cultures at the highest concentration of 15 μM F_3mdCyd. In contrast, DNA samples from uninfected, analogue-treated CEM cell cultures showed no detectable incorporated cpm above background (12 cpm) in HPLC fractions specific for F_3mdCyd, and low levels (10–45 cpm) in HPLC fractions specific for F_3dThd (data not shown).

Methylation increases in the DNA of HSV-1-infected CEM cells after F_3mdCyd treatment correlates with the HSV-1 antiviral effect

HSV-1-infected CEM cells were cultured for 72 h in the presence of increasing concentrations of F_3mdCyd to determine if incorporation of F_3mdCyd into cellular DNA increased the extent of DNA methylation. DNA was then extracted for high performance liquid chromatography (HPLC) analysis.

DNA methylation levels in persistently infected, F_3mdCyd treated CEM cells exhibited dosimetric increases in F_3mdCyd incorporation into DNA after treatment with increasing concentrations of F_3mdCyd (Fig. 4). Determination of DNA methylation levels of persistently infected CEM cells, treated with 7.5 nM F_3dThd as a control analogue for the deamination of F_3mdCyd, was additionally performed. The appropriate equitoxic concentration of F_3dThd (1000-fold lower) used in treatment was determined by previous cell growth studies (data not shown). F_3dThd (as the deaminated product of F_3mdCyd) is an analogue of thymidine, and presumably should not act as a methylation signal to methyltransferase. DNA from these F_3dThd-treated CEM cells, exhibited background levels of 3.0% $\pm$ 0.30 methylation (Fig. 4).

Higher levels of DNA methylation correlated with loss of productive virus as shown by plaque assay (Fig. 5). HSV-1 viral titers (PFU/ml) of infected, untreated CEM cell cultures decreased approximately 2 logs (from log 2.8 $\pm$ 0.08 to log 0.6 $\pm$ 0.06) after 72 h treatment with F_3mdCyd when HSV-1-infected CEM cell cultures were treated at 15 μM F_3mdCyd with 20 μM H_4dUrd (data not shown).

It was also of interest to determine if the antiviral effects of F_3mdCyd were transient after removal of the analogue from culture. A second regimen of treatment included 72 h of analogue treatment followed by thorough washing of cell cultures, and medium replacement with or without analogue.

When virus was inhibited by treatment of CEM cell cultures with 7.5 μM F_3mdCyd for 72 h, the extent of DNA methylation was highest at 8.6 $\pm$ 0.8% compared to control persistently infected, untreated CEM cell cultures, which

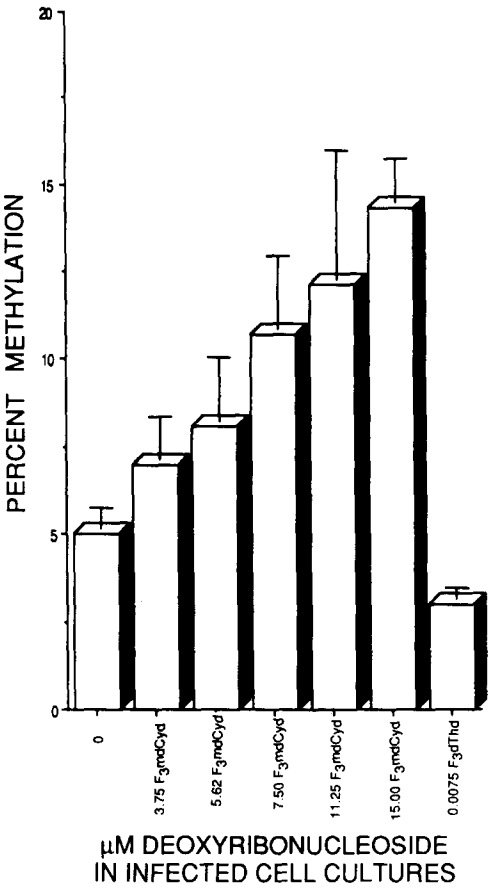

Fig. 4. The extent of DNA methylation in persistently infected CEM cells after treatment with F_3mdCyd and H_4dUrd for 72 h. Persistently infected CEM cells were incubated for 72 h with various concentrations of F_3mdCyd with 20 μM H_4dUrd or F_3dThd (as indicated) in the presence of [^{3}H]dCyd for the isotopic labeling of dCyd and mdCyd. DNA was then extracted and purified for analysis by HPLC. During HPLC analysis, fractions were collected and counted for radioactivity. Values represent mean percent DNA methylation $\pm$ SE of at least three determinations. Percent DNA methylation was calculated by the following equation: percent DNA methylation = $\frac{\text{pm (mdCyd)}}{\text{pm (mdCyd) + pm (dCyd) + pm (F_3mdCyd).}}$

exhibited DNA methylation of 3.2 $\pm$ 0.1% (Fig. 5A). These higher levels of DNA methylation (8.6%) observed after treatment of CEM cell cultures with 7.5 μM F_3mdCyd for 72 h, were coincident with a decrease in HSV-1 titers to $<1.0 \pm 0.1$ $\log_{10}$ PFU/ml (Fig. 5B) compared to control HSV-1-infected, untreated CEM cell cultures which possessed HSV-1 titers of 3.9 $\pm$ 0.2 $\log_{10}$ PFU/ml. However, the extent of DNA methylation again decreased to baseline levels of 3.7 $\pm$ 0.4%, after F_3mdCyd was washed from CEM cell cultures, not replaced and the cell cultures incubated for an additional 5 days (Fig. 5A). This

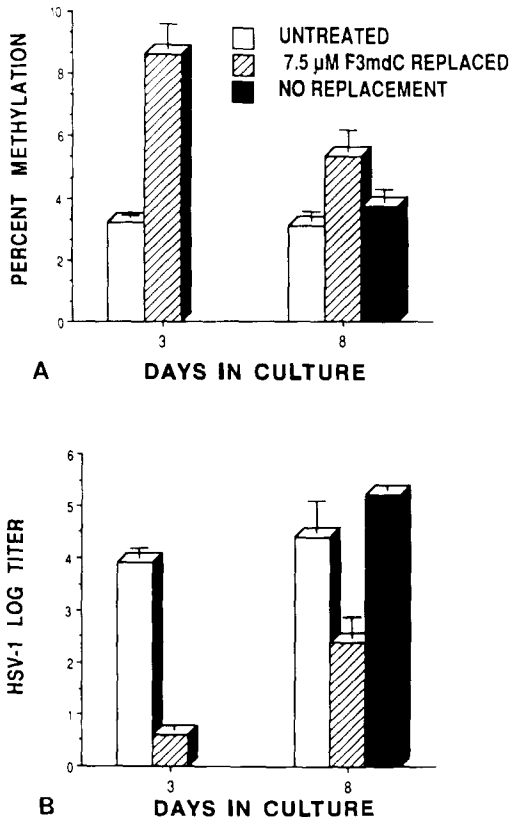

Fig. 5. Comparison of the extent of DNA methylation with HSV-1 titers of persistently infected CEM cells after treatment with 7.50 μM F_3mdCyd and 20 μM H_4dUrd for 72 h, followed by analogue replacement or removal from cultures. Persistently infected CEM cultures were treated with 7.50 μM F_3mdCyd and 20 μM H_4dUrd for 72 h. The cell culture was then split and the analogue was replaced at the same concentration. The analogue was washed from replicate cell cultures. After 5 days of continued growth, cell cultures were harvested to determine the percent methylation by HPLC, as well as the HSV-1 titer. **A**: Percent DNA methylation of CEM cells at day 3 (post analogue treatment) and day 8 (post analogue treatment followed by analogue replacement or removal from cultures). Values represent mean percent DNA methylation + SE of at least three separate determinations. **B**: HSV-1 $\log_{10}$ PFU/ml of CEM cells at day 3 (post analogue treatment) and day 8 (post analogue treatment followed by analogue replacement or removal from cultures). Values represent the mean of duplicate plaque assays + SE of at least two separate determinations.

decrease in the extent of DNA methylation was coincident with the rise of HSV-1 titers to 5.2 $\pm$ 0.1 $\log_{10}$ PFU/ml (Fig. 5B). When 7.5 μM F_3mdCyd was re-added to persistently infected CEM cell cultures for an additional 5 days after the initial 72-h analogue treatment period, the extent of DNA methylation remained higher (5.3 $\pm$ 0.7%) than that of control persistently infected, untreated CEM cell cultures (3.1 $\pm$ 0.3%) (Fig. 5A), while HSV-1 titers in

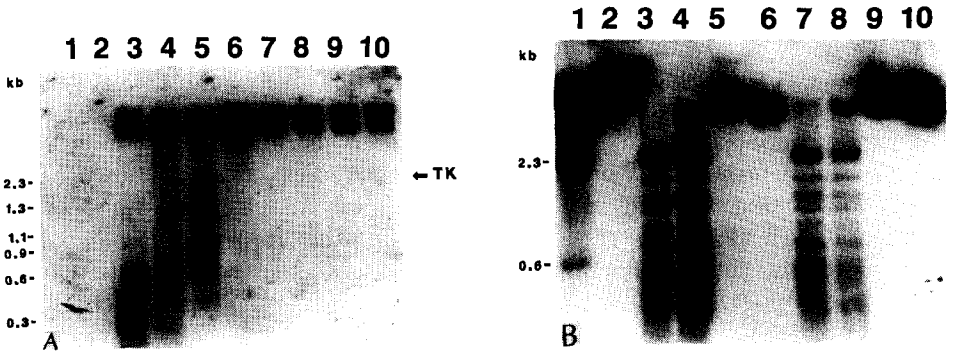

Fig. 6. Autoradiograph of a Southern blot of DNA from Vero cells probed with ^{32}P-labeled total genomic HSV-1 DNA. **A**: 5 μg DNA from HSV-1-infected, untreated and 10 μg DNA from HSV-1-infected, analogue-treated Vero cells was extracted and purified for restriction digestion. 1 μg of lambda DNA was added to each sample of Vero cell DNA (as an internal control for the completeness of restriction digestion). These samples were digested with 5–10 × the required restriction enzyme units to ensure complete digestion, electrophoresed in a 1.5% agarose gel, blotted and probed with ^{32}P-labeled total genomic HSV-1 DNA. Lane 1: lambda/*Hin*dIII and ϕX174/*Hae*III molecular-weight markers; lane 2: DNA from uninfected and undigested Vero cells; lane 3: DNA from HSV-1-infected, untreated Vero cells digested with *Hpa*II; lane 4: DNA from HSV-1-infected, untreated Vero cells digested with *Msp*I; lane 5: DNA from HSV-1-infected, untreated Vero cells digested with *Sma*I; lane 6: DNA from HSV-1-infected, untreated Vero cells digested with *Eco*RI; lane 7: DNA from HSV-1-infected Vero cells treated with 1.2 mM F_3mdCyd/200 μM H_4dUrd and digested with *Hpa*II; lane 8: DNA from HSV-1-infected Vero cells treated with 1.2 mM F_3mdCyd/200 μM H_4dUrd digested with *Msp*I; lane 9: DNA from HSV-1-infected Vero cells treated with 1.2 mM F_3mdCyd/200 μM H_4dUrd digested with *Sma*I; lane 10: DNA from HSV-1-infected Vero cells treated with 1.2 mM F_3mdCyd/200 μM H_4dUrd digested with *Eco*RI. **B**: Autoradiograph of reprobing of Southern blot with ^{32}P-labeled lambda DNA. The blot shown in (A) was stripped and reprobed with ^{32}P-labeled lambda DNA (to verify the complete digestion of sample DNAs). Details of gel loading as above.

CEM cell cultures in which analogue was re-added remained lower than those of control-untreated CEM cell cultures at 2.4 $\pm$ 0.4 $\log_{10}$ compared to 3.9 $\pm$ 0.2 $\log_{10}$, PFU/ml, respectively (Fig. 5B).

Southern blot analyses of HSV-1 DNA from infected Vero cells during the time of HSV-1 inhibition by F_3mdCyd

To determine if F_3mdCyd was specifically incorporated into the HSV-1 DNA of persistently infected cells, we used (as suggested by Dr. R. Desrosiers, personal communication) the Vero cell line, which is able to generate high levels of infectious virus of 10^7–10^8 PFU/ml, to facilitate the detection of F_3mdCyd into HSV-1 DNA by Southern blotting. In contrast, persistently infected CEM cells possess low copy numbers of viral genomes (1–3 copies/cell) (Youssouffian et al., 1982) which are further reduced after treatment with the analogue, and were, therefore, unsuitable for these analyses.

In standard treatment procedures, DNA analogues are added either at the time of viral adsorption, or 1 h after viral adsorption. To assess the effects of

analogue treatment on the methylation status of HSV-1 DNA, total DNA was extracted 18–24 h post viral adsorption from HSV-1-infected Vero cells which were treated with 1.2 mM F_3mdCyd and 200 μM H_4dUrd at 6 h post viral adsorption (as suggested by Dr. N. Fregien, University of Miami School of Medicine, FL), as well as from control HSV-1-infected, untreated Vero cells. The optimal concentration of F_3mdCyd used to maintain >80% Vero cell viability was determined by previous cell growth studies (data not shown). This incubation period allowed the virus to complete at least one third of its replication cycle (Roizman et al., 1967). By using this approach, the increased numbers of viral copies resident in the infected Vero cell after 6 h would then be able to incorporate analogue and thus result in a significant increase in detection of HSV-1 sequences by Southern blotting.

DNA from uninfected, undigested Vero cells showed no detectable hybridization with the genomic HSV-1 DNA probe after washing blots at medium and high stringencies (Fig. 6A; lane 2) indicating that HSV-1 probe hybridization was specific for HSV-1 with no detectable cross-hybridization with Vero cell DNA. After digestion with *Hpa*II, *Msp*I, *Sma*I and *Eco*RI, DNA from HSV-1-infected, untreated Vero cells consistently revealed the presence of low-molecular-weight fragments ranging from <300 to approximately 2400 bp (Fig. 6A; lanes 3,4,5,6, respectively). In contrast, these low-molecular-weight fragments were not evident in the DNA from HSV-1-infected, F_3mdCyd-treated Vero cells digested with the same enzymes (Fig. 6A; lanes 7,8,9,10, respectively). In all cases of HSV-1-infected, F_3mdCyd-treated Vero cell DNA which was digested with *Hpa*II, *Msp*I, *Sma*I and *Eco*RI, increases in methylation were observed by the presence of only 2 high-molecular-weight bands (Fig. 6A; lanes 7,8,9,10, respectively). There were no observable differences in digestion by methyl-sensitive (*Hpa*II) vs. methyl-insensitive (*Msp*I) isoschizomers of the DNA from HSV-1-infected, F_3mdCyd-treated Vero cells (Fig. 6A; lanes 8,9). Inhibition of both *Hpa*II and *Msp*I digestion was previously observed in our studies using mdCyd and F_3mdCyd hemisubstituted M13 DNA (Fig. 1B), and appears to reflect the substitution of F_3mdCyd at both cytosines of the CCGG recognition sequence.

An interesting detail observed in these digests was the appearance of high-molecular-weight (in addition to the expected low-molecular-weight) fragments in the *Hpa*II digest of DNA from untreated, HSV-1-infected, untreated Vero cells (Fig. 6A; lanes 4,5) which is uncharacteristic for the restriction pattern of HSV-1 virion DNA. These high-molecular-weight fragments seen in the *Hpa*II and *Msp*I digest of DNA from HSV-1-infected Vero cells could not be the result of incomplete digestion of the Vero cell DNA since the lambda DNA used as an internal control for restriction digestion (Fig. 6B; lanes 3,4,7,8) is clearly digested to completion by *Hpa*II and *Msp*I. It is possible that the high-molecular-weight bands which are observed in DNA from HSV-1-infected, untreated Vero cells (Fig. 6A; lanes 3,4) represent a portion of intracellular HSV-1 DNA which remains methylated during productive infection of Vero cells (see Discussion). Lambda DNA is not an adequate control for digestion

by *Sma*I or *Eco*RI because there are only 3 and 5 restriction sites in lambda DNA for these enzymes, respectively. Due to the high percent (1.5%) agarose gel used for electrophoresis, the large-molecular-weight fragments in lambda DNA revealed after digestion with *Sma*I and *Eco*RI, are not well resolved. As a control for undigested DNA, λDNA was included in the control blot of Fig. 6B; lane 2.

Discussion

We have used two different systems to analyze the molecular and cellular effects of 5-halogenated dCyd analogue incorporation into DNA.

F_3mdCyd effects on eukaryotic CEM (T-lymphoblastoid) methyltransferase activity (as well as similar methylation sensitive restriction enzymes) were determined using a synthetic analogue hemisubstituted M13 DNA construct.

The restriction enzymes selected for analyses were the isoschizomers *Hpa*II (Sharp et al., 1973) and *Msp*I (Waalwijk and Flavell, 1978). *Hpa*II has the same recognition sequence as *Msp*I (CCGG). Unlike *Msp*I, however, *Hpa*II fails to cleave when the inner cytosine of the sequence is methylated. *Msp*I will cleave when the inner cytosine of the sequence is methylated, but fails to cleave when the outer, or both cytosines of the sequence is methylated (Keshet and Cedar, 1983).

Additionally, the presence of competitive dCTP in the reaction mixture with F_3mdCTP during M13 primer extension showed that dCTP competes with F_3mdCTP for incorporation into M13 DNA. This fact was evident when comparative increases (Fig. 2) or decreases (Fig. 1) in dCTP vs. F_3mdCyd concentration affected dCTP incorporation into M13 DNA by showing concomitant decreases (Fig. 1) or increases (Fig. 2) in the cleavage of M13 DNA by *Hpa*II and *Msp*I. From these results, we infer that substitution of DNA by F_3mdCTP is specifically at dCTP sites.

We conclude that F_3mdCyd in DNA acts in a very similar manner to incorporated 5-methyldeoxycytidine as a methylation signal to restriction enzymes possessing CG recognition sites and impairs digestion when the hemisubstituted M13 DNA substrate is fully substituted at all dCyd sites. No digestion by either *Hpa*II or *Msp*I of the hemisubstituted M13 DNA construct is observed when no dCTP is present and all dCyd sites are substituted with mdCTP or F_3mdCTP during primer extension. Extensive digestion by both *Hpa*II and *Msp*I of the hemisubstituted M13 construct is observed when the concentration of mdCTP or F_3mdCTP is present during primer extension at only 10% the concentration of competitive dCTP, and a large proportion of the available dCyd sites are substituted with dCyd.

Using uninfected CEM cells as the source for eukaryotic methyltransferase, we additionally showed that after F_3mdCyd is incorporated into DNA, the analogue acts as an efficient template for methyl modification of DNA. The ability of F_3mdCyd hemisubstituted M13 to act as a template for CEM

methyltransferase was very similar to that of mdCyd hemisubstituted M13 DNA, and was significantly increased when compared to dCyd hemisubstituted M13 DNA as template.

Further studies were pursued in a CEM cell line persistently infected with HSV-1 to study DNA methylation changes both during the presence or absence of F_3mdCyd in culture. Incorporation of F_3mdCyd, without deamination to F_3dThd, was shown to occur only in HSV-1-infected, but not uninfected CEM cultures. From these experiments we were able to show that F_3mdCyd, but not F_3dThd, is incorporated at significant levels into the DNA of HSV-1-infected CEM cells when deamination is prevented at the nucleoside and nucleotide levels with H_4dUrd. Incorporation of F_3mdCyd does not occur to any significant extent in the DNA of uninfected, analogue-treated CEM cells.

The reduction of HSV-1 titres in infected CEM cell cultures by treatment with F_3mdCyd correlated with its incorporation into DNA of infected CEM cells as well as with increased levels of methylation of DNA of infected CEM cells. These data support a correlation between inhibition of replication of HSV-1 and the presence of hypermethylation of DNA in persistently infected CEM cells. HSV-1 inactivation occurs coordinately with increases in DNA methylation. However, when the analogue is removed from cultures, the decrease of infectious viral particles appears, from a population standpoint, to be a reversible phenomenon, with HSV-1 titers again rising at a time when hypomethylation is observed. This effect is contradictory to the well-described maintenance methylation properties of DNA methyltransferase (Riggs, 1975; Holliday and Pugh, 1975).

Possible reasons for the apparent lack of methylation maintenance after incorporation of F_3mdCyd into DNA may be the extrachromosomal location of HSV-1 which eludes modification by cellular methyltransferase (a mechanism previously described for Polyoma virus (Subramanian, 1982) and SV40 (Yisraeli, 1981)) as well as the exponential levels of viral vs. cellular replication. Rapid replication of HSV-1 may overwhelm the modification capacity of DNA methyltransferase which remains closely associated with the bulk of chromosomal DNA (Burdon et al., 1985). A likely possibility is that there may be a subpopulation of infected CEM cells in which incorporation of analogue into DNA was low, resulting in a selection of virus which eluded the effects of analogue treatment.

DNA from HSV-1-infected, F_3mdCyd-treated Vero cells, was not cleaved by either *Hpa*II and *Msp*I although unmethylated λDNA (included as an internal digestion control) was completely digested by *Hpa*II and *Msp*I. Additionally, high- and low-molecular-weight fragments were observed in the *Hpa*II digest of DNA from HSV-1-infected, untreated Vero cells.

Sharma and Biswal (1977), made the initial discovery that intracellular herpesvirus DNA becomes partially methylated approximately 6–8 h after infection. This condition was described as 'transient' because the fully mature, extracellular HSV-1 virion DNA contains no detectable methylation (Sharma and Biswal, 1977; Low et al., 1969). Since this observation was made by

Sharma and Biswal in 1977, no other experimental work has explored this interesting phenomenon. It is possible that the high-molecular-weight bands which are observed in DNA from HSV-1-infected, untreated Vero cells (Fig. 6A; lanes 3,4) represent a portion of intracellular HSV-1 DNA which remains methylated during productive infection of Vero cells. Alternatively, it is also possible that these high-molecular-weight bands which are observed in DNA from HSV-1-infected, untreated Vero cells (Fig. 6A; lanes 3,4) represent some level of non-specific cross-hybridization which occurred between the digested Vero cell DNA within the HSV-1-infected cell with the genomic HSV-1 DNA probe. Peden et al. (1982) and Puga et al. (1982) have described the existence of homology between mammalian cell DNA sequences and several viral genomes in the family of Herpesvirus. But it should also be noted that specific HSV-1 DNA hybridization occurred when the genomic HSV-1 DNA probe for Southern blotting was used (shown by the typical restriction patterns for HSV-1 DNA by *Hpa*II, *Msp*I, *Sma*I and *Eco*RI) (Wigler et al., 1977; Youssoufian et al., 1982). Yet another possibility is that HSV-1 sequences become integrated into Vero cell DNA, giving rise to hybridization of flanking cellular sequences when probed with the total genomic HSV-1 DNA probe. The methyl-like effects of incorporated F_3mdCyd on restriction digestion are also likely to occur concomitantly in Vero cell DNA residing within the HSV-1-infected, analogue-treated Vero cell. In either or both cases, the inhibition of restriction digestion by the presence of incorporated F_3mdCyd is clearly evident in the total DNA of HSV-1-infected, analogue-treated Vero cells.

From these results, we conclude that F_3mdCyd is incorporated into the DNA of HSV-1 (as well as cellular DNA) within the infected cell, resulting in the inhibition of restriction enzyme digestion in a manner similar to the effects of 5-methyldeoxycytidine incorporation. This methylation-like property of the incorporated analogue occurs concomitantly with, and appears to be at least one of the mechanisms involved in the analogue's antiviral effects towards HSV-1. Although studies from our laboratory have shown that thymidine antagonizes the HSV-2 antiviral effect of F_3methyldCyd when coadministered with H_4Urd (Fox, 1979), the antagonism could be at the level of competition for phosphorylation at the nucleoside and nucleotide levels. Thus, while the reversal by thymidine of viral inhibition by F_3mdCyd is consistent with antagonism of the incorporation of F_3dThd formed from the deamination of F_3mdCyd, it does not exclude a mechanism in which inactivation of the virus is due to changes in the methylation status of cytosine in the DNA of virus infected cells.

Several distinct lines of investigation, including past studies from our laboratory, have suggested a possible approach to the problems of herpesvirus reactivation in recurrent infections, utilizing agents such as BrdCyd and IdCyd, as well as F_3mdCyd, which have the potential to act as selective hypermethylating agents.

The findings in these separate lines of study are as follows:

- 5-BrdCyd, 5-IdCyd, and 5-F_3mdCyd are selectively phosphorylated by the

pyrimidine nucleoside kinase encoded by HSV-1 and HSV-2 (Cooper, 1973; Schildkraut et al, 1975; Dobersen and Greer, 1978; Fox et al., 1983);

- the 5-halogenated analogues of deoxycytidine are less cytotoxic than the uridine analogues only when the dCyd analogues are utilized with an inhibitor of deamination (Shildkraut et al., 1975; Fox et al., 1979, 1982; Mekras et al., 1985). In contrast, the antiviral effect of the 5-halogenated analogues of deoxycytidine in cell culture is essentially equal to that of the deoxyuridine analogues when deamination is prevented or when an inhibitor of deamination is omitted (Shildkraut et al., 1975; Fox et al., 1979, 1982). For example, IdCyd possesses potent antiviral activity when deamination is prevented by inhibiting the deaminating enzymes or by use of 4-*N* alkyl substituted analogues of IdCyd (Fox et al., 1983);
- 5-BrdCyd, 5-IdCyd, and 5-F_3mdCyd (as shown in the present studies) are incorporated, as such, without deamination to the thymidine analogue into the DNA of herpesvirus infected cells (Fox et al., 1983).
- CEM cells, persistently infected with HSV-1, are hypomethylated during the viral productive state and hypermethylated during the spontaneous latent state as determined by the use of isoschizomer analyses of HSV-1 DNA (Youssoufian et al., 1982).
- 5-azacytidine and other DNA hypomethylating agents have been shown to reactivate latent herpesvirus in guinea pig and mouse neural tissues (Bernstein and Kappes, 1988; Stephanopoulos et al., 1988; Whitby et al., 1987).
- 5-BrdCyd and 5-IdCyd are comparable to 5-mdCyd in acting as a signal for methylation towards methylation-sensitive restriction enzymes and eukaryotic methyltransferases (Hardy et al., 1988).

In the present study, these properties are shown to be characteristic of 5-F_3mdCyd as well. Specificity of methylated sites has not been established, nor have we yet conducted experiments to determine effects on gene expression, a compelling next step in our studies. Our observations, however, suggest there may be direct correlations between methylation states resulting from the incorporation of F_3mdCyd into DNA and the antiviral effect against HSV-1. These sites of methylation may not be extensive (Dressler et al., 1987) but, if instrumental in viral genetic regulation, may be key to inactivation of the virus. Taken together, these lines of study suggest a strategy in which one may obtain selective hypermethylation of herpesvirus DNA and thereby affect the process of subsequent activation which leads to emergence of the virus. Additionally, since the HSV-1 genome is comprised of 70% CG nucleosides (McGeoch et al., 1989) the viral molecule possesses significant potential, not only as an excellent substrate for the substitution of a dCyd analogue, but also as an ideal substrate for methyl modification. We speculate that there is a definite function for the placement of these methylated residues, and that the progress of HSV-1 into either the productive or non-productive state may revolve upon a mechanism which acts to either diminish or perpetuate these methylated sites according to a temporal regulatory program.

It is possible that the process of activation of herpesvirus is unrelated to the

extent of methylation of its DNA. For example, viral activation may involve the inhibition of DNA methyltransferase, or the inhibition of SAM regeneration from any of its precursors. If this is the case, then it is unlikely that the extent of methylation of viral DNA during latent infection is a factor in preventing recurrent infections. However, if these mechanisms are not involved during the transition to the productive state of HSV-1 (or HSV-2), then our studies suggest that analogue mediated hypermethylation of the HSV-1 (or HSV-2) genome may affect the activation of the virus. For example, the presence of transcripts of an immediate early gene of HSV-1 (ICPO) are reported to be required for the reactivation of latent virus (Steiner et al., 1989; Hill et al., 1990). A similar example could be presented for the *lat* gene, which does not appear to be necessary for the establishment of latency but whose activity is required for activation (Javier et al., 1988; Tenser et al., 1989; Steiner et al., 1989; Krause et al., 1990; Hill et al., 1990). If ICPO or the *lat* gene was methylated and remained nonfunctional, the state of latency may be established and maintained but not be alterable by reactivation. If there is a limited reservoir of latent viruses and viruses from preceding active infections play a major role in contributing to subsequent recurrent infections, then our studies suggest an approach to control recurrent HSV infections. Thus, analogue-mediated hypermethylation of the herpes virus genome, which possesses no detectable methylation in its extracellular (virion) stage, may be an important mechanism in different aspects of herpes viral inactivation.

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