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Specifically Unmethylated Cytidylic-Guanylate Sites in *Herpesvirus saimiri* DNA in Tumor Cells

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The restriction endonucleases *Msp*I (CCGG), *Hpa*II (CCGG), *Fnu*DII (CGCG), and *Hae*III (GGCC) were used to study the methylation of *Herpesvirus saimiri* DNA in tumor cells taken directly from tumor-bearing animals. No evidence was found for methylation of the 5′ terminal C in the sequence CCGG or of the internal C in the sequence GGCC, but extensive methylation of CG was detected. Fifteen *Hpa*II sites and 17 *Fnu*DII sites were detected in the unique DNA region of the *H. saimiri* strain used. Twenty-eight of the 32 sites were methylated in greater than 90% of the viral DNA molecules in tumor cells, but the remaining 4 sites were unmethylated in greater than 95% of the viral DNA molecules in tumor cells. The locations of the four specifically unmethylated sites were mapped and appeared to be identical in the four different induced leukemias examined (one owl monkey and three white-lipped marmosets). The nonproducer 1670 tumor cell line, in continuous passage for over 7 years, contained four similar specifically unmethylated sites. Possibilities for the physiological significance of the unmethylated sites are discussed.

Herpesvirus saimiri naturally infects squirrel monkeys (*Saimiri sciureus*), in which it causes no apparent disease. Infection of other species of New World primates, however, frequently results in lymphoma or leukemia or both. Infectious virion DNA contains a high degree of intramolecular heterogeneity in guanine plus cytosine (G+C) content. The centrally located, unique DNA region is 115 kilobase pairs (kb) in length, includes the vast majority of coding sequences, and is 36% G+C (light DNA [L-DNA]). L-DNA has covalently attached at each end repetitive DNA with 71% G+C content (heavy DNA [H-DNA]). Each repeat unit of 1.3 kb is oriented in the same direction, and approximately 30% of an infectious virion DNA molecule is H-DNA (for review, see reference 9 and B. Fleckenstein and R. Desrosiers, *In* B. Roizman (ed.), *The Herpesviruses*, in press). The structure of infectious virion DNA (M-DNA) is shown schematically in Fig. 1.

5-Methylcytosine (m^5C) is the only modified residue that has been detected in mammalian cell DNA; m^5C occurs by far most frequently in the following palindromic sequence:

5′ CG 3′
3′ GC 5′

CG is the least common of the 16 dinucleotides, and various estimates have indicated that 50 to 90% of CG dinucleotides in mammalian cell DNA are methylated (3, 12).

Three lines of evidence indicate that *H. saimiri* virion DNA produced from lytic infection of cultured owl monkey kidney (OMK) cells is unmethylated or nearly so. (i) After hydrolysis, the nucleosides present in unlabeled, purified *H. saimiri* virion DNA have been analyzed by high-performance liquid chromatography (R. Desrosiers, J. Gehrke, and M. Ehrlich, unpublished data); m^5C was below detectable levels, at least 30 times less than the level in host cell (OMK) DNA. This is less than 20 methyl groups per L-DNA region. (ii) Acken et al. have labeled *H. saimiri* virion DNA with radioactive precursors and were also unable to detect either m^5C or m^6A (1). Their reported limits of detection indicate less than one methyl group per virion M-DNA molecule. (iii) Virion L- and H-DNA digested with *Hpa*II produced patterns that were indistinguishable from those of *Msp*I (6). The sequence specificities of *Hpa*II and *Msp*I are shown in Table 1. Greater than 95% of the CCGG sites in virion DNA appear to be by this criterion unmethylated; this represents less than one methylated CCGG per L-DNA region and less than 10 per H-DNA region.

Cell lines have been established from *H. saimiri*-induced tumors, and these have been used to study the persistence of the viral DNA. Viral DNA in cells of nonproducing lines has been previously reported to be extensively methylated. Since virus gene expression in these tumor cell lines is severely limited and since the level

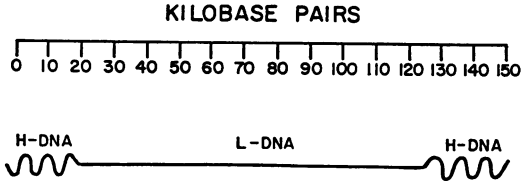

FIG. 1. Arrangement of unique L-DNA (36% G+C) and repetitive H-DNA (71% G+C) in infectious *H. saimiri* DNA. Each repeat unit in H-DNA is 1.3 kbp (2).

of viral DNA methylation is greatest in cell lines not producing virus, it was suggested that perhaps the methylation of viral DNA in tumor cells plays some role in limiting gene expression (6). The methylation status of a number of viral and cellular genes has since been analyzed, and the methylation of at least certain CG sites generally correlates inversely with expression in RNA (for review, see references 7 and 19). In this report, analyses are extended to *H. saimiri* DNA in tumor cells taken directly from tumor-bearing animals, and a few CG sites of many (4 of 32 so far examined) are described that specifically remain unmethylated in viral L-DNA in tumor cells.

MATERIALS AND METHODS

Cells and virus. The propagation of *H. saimiri* in OMK cells (line 637, passages 10 through 30) and purification of virus have been described previously (2). The 1670 cell line, originally established from an *H. saimiri*-induced marmoset tumor, was grown in RPMI 1640 medium containing 10% fetal calf serum (5, 16). Tumor cells were obtained directly from peripheral blood of animals with leukemia induced by *H. saimiri* strain S295C. Blood was drawn 3 to 14 days before death in the four different animals used in this study. The four animals included one owl monkey (*Aotus trivirgatus*; death at 12 months) and three white-lipped marmosets (*Saguinus fuscicollis-nigricollis*; death at 2 to 3 months). Leukocyte counts at the time DNA was prepared ranged from 20,000 to 200,000 per mm^3. Tumor cells were prepared by banding over Ficoll-Hypaque (density = 1.076 g/cm^3).

Analysis of DNA. Purified virion DNAs and tumor cell DNAs were prepared as described previously (2, 5). Aliquots containing 2 μg of DNA were digested with restriction endonucleases (New England Biolabs), as indicated in each figure, and electrophoresed through a 0.6% agarose gel. Control digestions of virion DNA contained 1 to 3 ng of the virion DNA plus 2 μg of OMK cell DNA. Digestions were performed for 4 to 20 h, with at least twice the amount of enzyme than was required to digest the DNA to detectable completion in 15 min. DNA fragments in agarose gels were transferred to nitrocellulose by the procedure of Southern (20), with acid nicking of the DNA in the gel before denaturation (5). Nicking of the DNA allows quantitative transfer even of high-molecular-weight DNA fragments. Transferred DNA was hybridized with virion L-DNA labeled with [^{32}P]dTTP in vitro by nick repair, rinsed extensively, and placed over Kodak X-Omat film for autoradiography (5). In the experiments shown in Table 2, lambda DNA (0.5 μg) was also included with each DNA sample for digestion; subsequent hybridization to ^{32}P-labeled lambda DNA demonstrated the completeness of each digestion. The fragment used for hybridization (see Fig. 4) was purified by electroelution from an agarose gel.

Tumor cells taken directly from peripheral blood of leukemic animals contained 50 to 300 viral genome equivalents per cell; this is similar to the number contained in most continuous tumor cell lines, but more than that found in lymph nodes or spleens of animals with diffuse lymphoma (10, 14). Lymph nodes and spleens of tumor-bearing animals undoubtedly contain a significant proportion of nontumor, virus-free cells. Peripheral leukocytes, after leukemia was induced by *H. saimiri* S295C, provided a rich and convenient source of essentially pure tumor cells.

TABLE 1. Sequence specificities of restriction endonucleases *Hpa*II and *Msp*I[a]

Sequence	*Hpa*II	*Msp*I
CCGG	+	+
CMGG	−	+
MCGG	+	−
MMGG	−	−

[a] The ability or inability to cleave the indicated sequence is noted by a + or −, respectively; M, 5-methylcytosine.

RESULTS

Degree of methylation. Methylation of CCGG sites was examined in *H. saimiri* DNA of six tumor cell lines and four fresh tumor samples by using *Hpa*II and *Msp*I. *H. saimiri* L-DNA in

TABLE 2. Degree of methylation of *H. saimiri* DNA in tumor cells

Origin of cells[a]	Producer vs nonproducer[b]	Degree of methylation[c]	
		L-DNA	H-DNA
1670	NP	1	1
70N2	NP	1	2
1926	P	2	4
77/5	P	3	4
H1591	P	2	4
MLC-1	P	3	4
Owl monkey tumor	P	1	4

[a] Listed here are five tumor cell lines, an in vitro transformed cell line (H1591), and tumor cells direct from a tumor-bearing owl monkey.

[b] Ability to produce virus when 10^6 cells were cocultivated with permissive OMK cells. NP, Nonproducer; P, producer.

[c] 1, >85% of CG sites methylated; 2, 50 to 85% of CG sites methylated; 3, 15 to 50% of CG sites methylated; and 4, <15% of CG sites methylated.

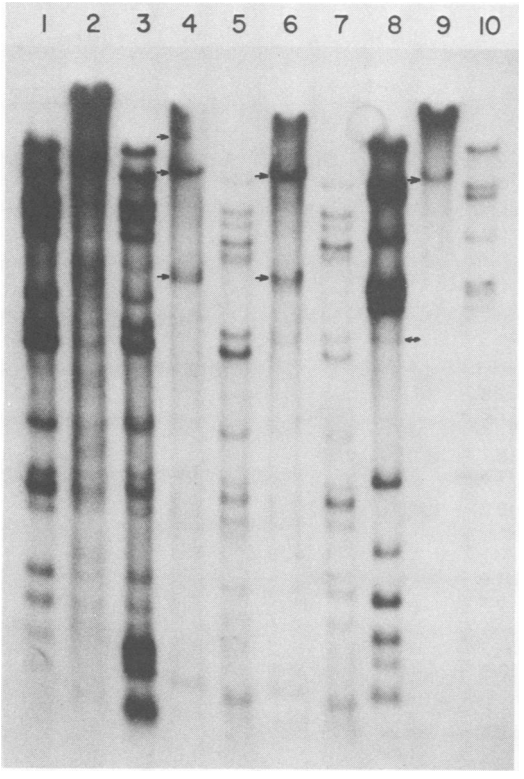

FIG. 2. Degree of methylation of *H. saimiri* L-DNA in tumor cells. DNA from tumor cells, tumor cell lines, and virions was digested with restriction endonuclease, electrophoresed overnight through a 0.6% agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled L-DNA of strain 11. Slot 1, strain 11 virion (*Hpa*II); slot 2, 1926 cell line (*Hpa*II); slot 3, 1926 cell line (*Msp*I); slot 4, 1670 cell line (*Hpa*II); slot 5, 1670 cell line (*Msp*I); slot 6, 70N2 cell line (*Hpa*II); slot 7, 70N2 cell line (*Msp*I); slot 8, strain S295C virion (*Hpa*II); slot 9, strain S295C-induced tumor (*Hpa*II); and slot 10, strain S295C-induced tumor (*Msp*I).

cells taken directly from a leukemic animal yielded the same *Msp*I fragments found in the parental strain S295C virion DNA (compare slots 8 and 10 in Fig. 2). The relative intensity of fragments also did not differ detectably. Thus, little if any methylation of the 5′ terminal C in the sequence CCGG occurred in *H. saimiri* L-DNA in tumor cells. Similarly, the *H. saimiri* L-DNA fragments produced from *Hae*III digestion of virion DNA and tumor DNA were indistinguishable (data not shown); this indicates little or no methylation of the internal C in the sequence GGCC (15). Very little digestion of *H. saimiri* L-DNA contained within the tumor DNA samples occurred with *Hpa*II; most of the hybridizing sequences were contained in material greater than 40 kb (Fig. 2, slot 9). This is indicative of extensive methylation of the internal C in the sequence CCGG. Note that a specific *Hpa*II L-DNA fragment of 17.5 kb was present in tumor DNA that did not comigrate with any parental virion DNA *Hpa*II fragment (upper arrow in slot 9 of Fig. 2). Such a fragment could arise from noncontiguous CCGG sites that were specifically not methylated; additional experiments presented below confirm this interpretation. Analyses of *H. saimiri* DNA contained within the three other tumor samples yielded results indistinguishable from those shown in Fig. 2 (slots 9 and 10), including the size and intensity of the specific 17.5-kb *Hpa*II fragment.

Methylation analyses of *H. saimiri* DNA contained within tumor cell lines were slightly more complicated due to the deletions, rearrangements, and duplications that are sometimes observed in these continuously passaged cells (5). *H. saimiri* L-DNA in the producing cell line 1926 (derived from an *H. saimiri* strain 11-induced tumor) yielded the same *Msp*I fragments that were found in the parental virion DNA (compare slots 1 and 3 in Fig. 2). The intensity of some of the L-DNA *Msp*I fragments in 1926 cells differed from virion DNA; this more than likely was due to duplication of some regions of the genome or populations of defective DNA molecules that arose through continued passage of the cell line. *Hpa*II digestion products of L-DNA in 1926 cells were very heterogeneous in size, with most L-DNA still of high molecular weight (Fig. 2, slot 2). Digestion of DNA from other producing cell lines with *Hpa*II and *Msp*I indicated similar levels of L-DNA methylation (Table 2).

H. saimiri L-DNA in the nonproducing tumor cell lines 1670 and 70N2 yielded some of the *Msp*I fragments that were found in the parental virion DNA (strain 11), but others were missing (compare slots 5 and 7 with slot 1 in Fig. 2). Previous work has demonstrated a 21-kb deletion in at least the majority of *H. saimiri* DNA molecules in 1670 and 70N2 cells (5, 23). Since we do not yet have a map of virion DNA *Msp*I fragments, we do not know whether the missing *Msp*I fragments are in fact derived from the region known to be deleted in 1670 and 70N2 cells, but the size of missing and new fragments is sufficient to be accounted for by the known 21-kb deletion. *Hpa*II digestion of 1670 and 70N2 cell DNA revealed the presence of high-molecular-weight *H. saimiri Hpa*II L-DNA fragments and also specific *H. saimiri* L-DNA *Hpa*II fragments that did not comigrate with any virion DNA *Hpa*II fragments and that did not comigrate with any L-DNA *Msp*I fragments found in 1670 or 70N2 cells (Fig. 2, slots 1, 4, and 6). Again, evidence presented below confirms that the specific *Hpa*II fragments arise

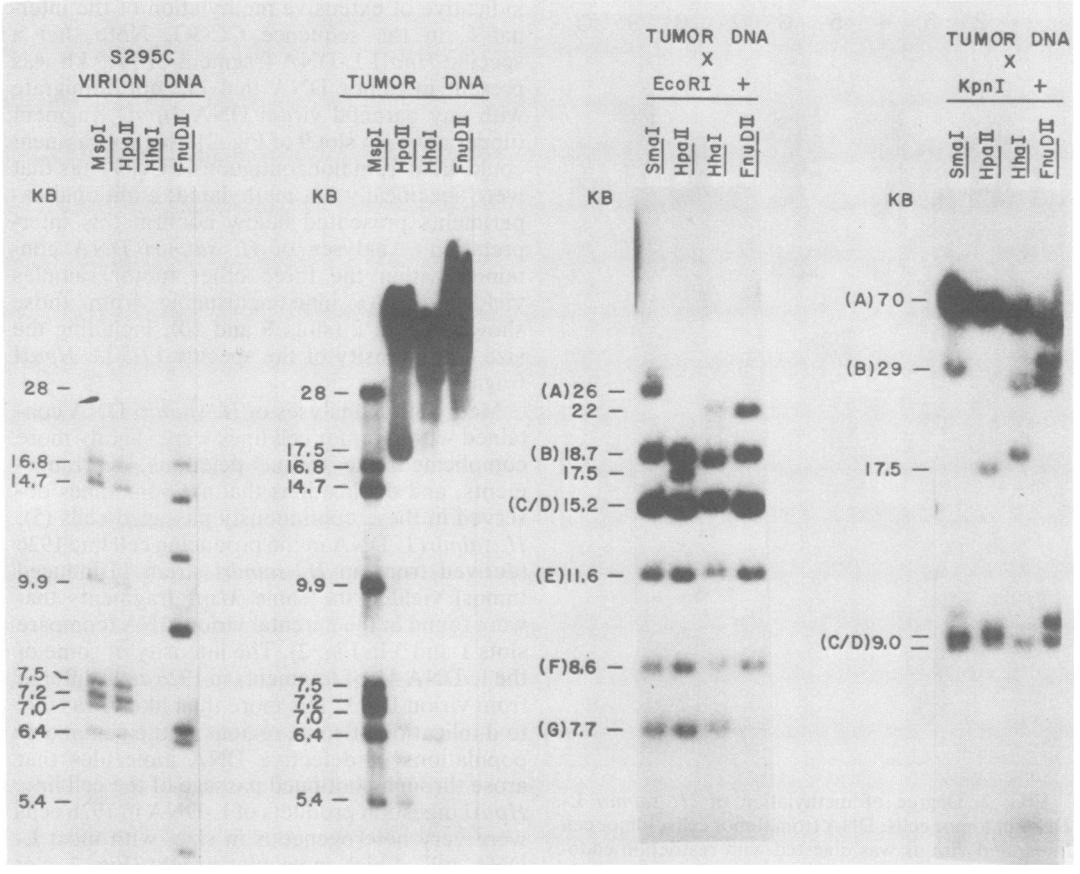

FIG. 3. Specifically unmethylated sites in tumor L-DNA. DNA was digested with restriction endonuclease(s), electrophoresed for 40 h through a 0.6% agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled L-DNA of strain S295C. A composite photo of autoradiograms from two different gels is shown. The restriction endonuclease *Sma*I cleaves three times in each repeat unit of strain S295C H-DNA, but does not cleave in L-DNA (C. Mulder and B. Fleckenstein, unpublished data). Since *Eco*RI, *Kpn*I, and *Bam*HI cleave only in L-DNA, additional *Sma*I digestion was used to reveal L-DNA terminal fragments as specific bands on these gels (*Eco*RI fragments A and J, *Kpn* fragments B and C, and *Bam*HI fragments A and E).

from noncontiguous CCGG sites that are specifically not methylated.

From the degree of digestion with *Hpa*II, the overall degree of CG methylation among CCGG sites was estimated (Table 2).

Virus was recovered from the producing cell lines 1926 and 77/5 by cocultivation with OMK cells, and purified virion DNA was analyzed by *Hpa*II and *Msp*I digestion. This virion DNA was not detectably methylated.

Mapping of specifically unmethylated CG sites in tumor cells. The specific *H. saimiri* L-DNA *Hpa*II fragments observed in DNA from fresh tumor cells and from nonproducing tumor cell lines (Fig. 2, slots 4, 6, and 9) suggested that specific CG sites were not methylated appreciably. It seemed necessary to confirm this interpretation and to map the location of specifically unmethylated sites. To achieve these goals, double digestion with *Hpa*II plus a restriction endonuclease whose cleavage sites in L-DNA are known was used.

For the analysis of methylated and unmethylated sites in L-DNA, it was desirable not to be confined to CCGG sites so other enzymes in addition to *Hpa*II were used. Methylation-sensitive restriction endonucleases with a six-nucleotide recognition sequence such as *Sma*I, *Sac*II, or *Sal*I cleave *H. saimiri* L-DNA too infrequently (0 to 3 times) for a detailed analysis. *Hha*I (GCGC) does not cleave at GMGC (where M = m^5C) (15), and results with this restriction endonuclease are reported below. *H. saimiri* DNA after stable association with a tumor cell was found to resist digestion by *Fnu*DII (CGCG) (Fig. 3). The inability to cleave most *Fnu*DII

TABLE 3. Summary of *H. saimiri* S295C L-DNA fragments found in tumors

Enzyme(s)	Fragments[a]	Fragments
*Hpa*II		>40, 17.5, 5.4 het
*Eco*RI	A, B, C, D, E, F, G, H, I, J, K	
*Eco*RI-*Hpa*II	B, C, D, E, F, G, H, I, J, K	17.5, 5.4 het, 2.5
*Kpn*I	A, B, C, D	
*Kpn*I-*Hpa*II	A, C, D	17.5, 5.4 het, 5.0
*Bam*HI	A, B, C, D, E, F	
*Bam*HI-*Hpa*II	B, C, D, E, F	17.5, 11.8, 5.4 het
*Fnu*DII		>40, 3.6 het
*Eco*RI-*Fnu*DII	B, C, D, E, F, H, I, J, K	22, 4.2, 3.4, 3.6 het
*Kpn*I-*Fnu*DII	C, D	~40, 33, 26, 3.6 het

[a] Letters refer to the *Eco*RI, *Kpn*I, or *Bam*HI fragments as shown in Fig. 6. The approximate sizes of fragments (in kb) are as follows: *Eco*RI-A, 26; *Eco*RI-G, 7.7; *Kpn*I-A, 70; *Kpn*I-B, 29; and *Bam*HI-A, 35 (reference 9).

[b] Numbers refer to the sizes of fragments (in kb) produced by *Hpa*II or *Fnu*DII digestion alone or the sizes of the unique fragments produced from the indicated double digestions. Het refers to the heterogeneity in these fragments (see Fig. 3 and text).

sites in viral DNA in tumor cells is taken as presumptive evidence for failure to cleave when at least one of the CG dinucleotides of its recognition sequence is methylated. As mentioned above, m^5C is the only modified nucleoside that has been detected in mammalian cell DNA, and no other reasonable explanation is available for the inability of a restriction endonuclease with CG in its recognition sequence to cleave many sites in viral DNA after stable association with a cell. The sequence specificities of *Hpa*II and *Msp*I have been well characterized, and the inability of *Hpa*II to cleave viral DNA in tumor cells is quite convincingly due to CG methylation. Nevertheless, the evidence with *Fnu*DII is presumptive until further data are forthcoming. Also, *Taq*I (TCGA) was found to produce the same viral DNA fragments in tumor cells that are found in purified virions (not shown); this is consistent with other recent work which found that *Taq*I cleaves whether or not the C of its recognition sequence is methylated (11, 21).

When ^{32}P-labeled strain S295C L-DNA was used for hybridization after Southern transfer of *Hpa*II-cleaved tumor DNA, a series of L-DNA fragments starting at 5.4 kb (5.4 kb, heterogeneous) was observed in addition to the specific 17.5-kb fragment and DNA >40 kb (Fig. 3). A strain S295C virion L-DNA fragment of 5.4 kb was not readily apparent in the blot shown in Fig. 2 (lower arrow slot 8) because of the use of ^{32}P-labeled strain 11 L-DNA for the hybridization. The left-most 7 kb of L-DNA from these two *H. saimiri* strains show little or no cross-homology (P. Medveczky, R. Desrosiers, E. Szomolanyi, L. Falk, and C. Mulder, unpublished data).

When tumor DNA was digested with both *Eco*RI and *Hpa*II, the left-terminal L-DNA *Eco*RI fragment A could no longer be detected (Fig. 3 and Table 3); the molarity of the other *Eco*RI fragments appeared to be unchanged. *Hpa*II also cleaves approximately seven times in each repeat unit of H-DNA. The results with *Hpa*II and *Eco*RI-*Hpa*II digestion shown in Fig. 3 and Table 3 were consistent with two specifically unmethylated CCGG sites within the *Eco*RI fragment A of L-DNA. Two lines of evidence suggested that 5.4-kb heterogeneous fragments were derived from the left-most region of the *Eco*RI fragment A: (i) the series of less-intense bands above the 5.4-kb *Hpa*II fragment suggested a variation in the amount of H-DNA attached to the 5.4-kb fragment due to heterogeneity of methylation in the repetitive H-DNA region immediately adjacent to L-DNA and (ii) the 5.4-kb *Hpa*II fragment in strain S295C virion DNA and the 5.4-kb heterogeneous *Hpa*II fragments in tumor DNA did not hybridize significantly when ^{32}P-labeled strain 11 L-DNA was used for the hybridization probe. The left-most 7 kb of L-DNA is the only region in which nonhomology between strains was detected. To further confirm the order of *Hpa*II sites within the viral L-DNA *Eco*RI fragment A in tumor cells, a *Sac*II fragment of 10 kb derived from the left end of strain S295C L-DNA was prepared, labeled in vitro, and used for hybridization (Fig. 4). As expected, the ^{32}P-labeled, left-terminal, 10-kb virion L-DNA fragment hybridized detectably only to the *Eco*RI fragment A of virion and tumor DNA. The left-terminal 10-kb fragment hybridized strongly to both the *Hpa*II 17.5-kb and the 5.4-kb heterogeneous fragments of tumor cells. This demonstrates that there are two CCGG sites in L-DNA of tumor cells, located approximately 5.4 and 22.9 kb from the left H-L DNA border, that are specifically not methylated. Double digestions with

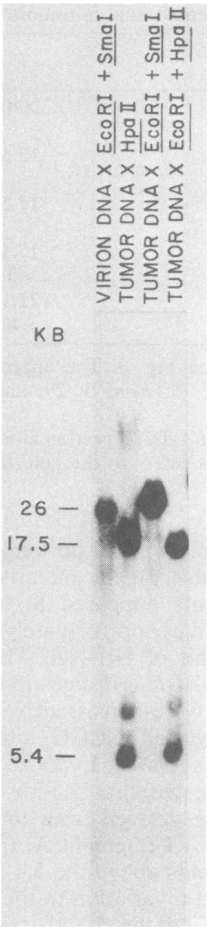

FIG. 4. Use of purified 10-kb left-terminal L-DNA fragment for hybridization. DNA was digested with restriction endonuclease(s), electrophoresed overnight through a 0.6% agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled *Sac*II fragment, which represents the left-most 10 kb of L-DNA.

*Kpn*I-*Hpa*II and *Bam*HI-*Hpa*II also confirmed this and did not reveal any other unmethylated CCGG sites (Fig. 3 and Table 3). (Cleavage maps of *H. saimiri* S295C L-DNA are shown at the top of Fig. 6.)

Similar experiments were performed with *Fnu*DII (Fig. 3 and Table 3). Double digestion of tumor DNA with *Eco*RI-*Fnu*DII revealed the presence of specifically unmethylated sites within the L-DNA *Eco*RI fragments A and G. The suspected locations of specifically unmethylated sites were confirmed by digestions with *Kpn*I-*Fnu*DII and *Bam*HI-*Fnu*DII (Fig. 3 and Table 3).

The data for *Hpa*II and *Fnu*DII presented in Fig. 3 and Table 3 represent a single tumor sample, but analyses of L-DNA contained within the other three tumor DNA samples produced indistinguishable patterns (data not shown).

Results with *Hha*I were not as straightforward. Although the *Eco*RI fragment A disappeared below detection after double digestion with *Eco*RI-*Hha*I, the intensity of some of the other fragments also appeared to decrease (Fig. 3). Furthermore, new fragments (e.g., *Eco*RI-*Hha*I, 22 kb) also appeared less than molar. There may be *Hha*I sites with intermediate levels of C methylation, and the use of cloned DNA fragments for hybridization and accurate quantitation will be required for a more detailed analysis.

Mapping of specifically unmethylated CG sites in 1670 cells. Digestion of 1670 cell DNA with *Hpa*II and *Fnu*DII and double digestion with *Eco*RI-*Hpa*II, *Eco*RI-*Fnu*DII, *Kpn*I-*Hpa*II, *Kpn*I-*Fnu*DII, *Bam*HI-*Hpa*II, and *Bam*HI-*Fnu*DII were used to map the location of specifically unmethylated sites in viral L-DNA in 1670 cells (Fig. 5 and other data not shown). The majority of viral DNA molecules in 1670 cells are composed of two segments of L-DNA (L1 and L2) separated by segments of H-DNA. L2 represents the left-most 54 kb of virion L-DNA, and L1 is approximately 92 kb, with 21 kb deleted from the central region of virion DNA (5, 23). When 1670 DNA was double digested with *Eco*RI-*Hpa*II, again only the left-terminal L-DNA *Eco*RI fragment A disappeared. The specific L-DNA *Hpa*II fragment of 17.5 kb in 1670 cells was not distinguishable in size by agarose gel electrophoresis from that found in fresh tumor DNA. The left-most *Hpa*II fragment in L-DNA of 1670 cells was found to be 7.2 kb, i.e., 1.8 kb larger than that found in tumor DNA. Double digestion with *Eco*RI-*Fnu*DII revealed a specifically unmethylated site within the *Eco*RI fragment A and within the 7.2-kb *Eco*RI fragment in 1670 cells, which includes sequences from virion *Eco*RI fragment G and spans the 21 kb of deleted sequences.

DISCUSSION

The analyses of fresh tumor and nonproducer cell line DNA described in this report have revealed the presence of a few CG sites in *H. saimiri* DNA that are specifically not methylated. Four *H. saimiri* S295C-induced tumors were analyzed, and four unmethylated CG sites that appeared identical in each of the four tumors were found. The locations of the four unmethylated CG sites in L-DNA of tumors are shown schematically in Fig. 6. Strain S295C virion L-DNA contains 15 *Hpa*II sites and 17 *Fnu*DII sites that produce fragments greater than 0.5 kb. In each of the tumor samples, two of the *Hpa*II sites and two of the *Fnu*DII sites were

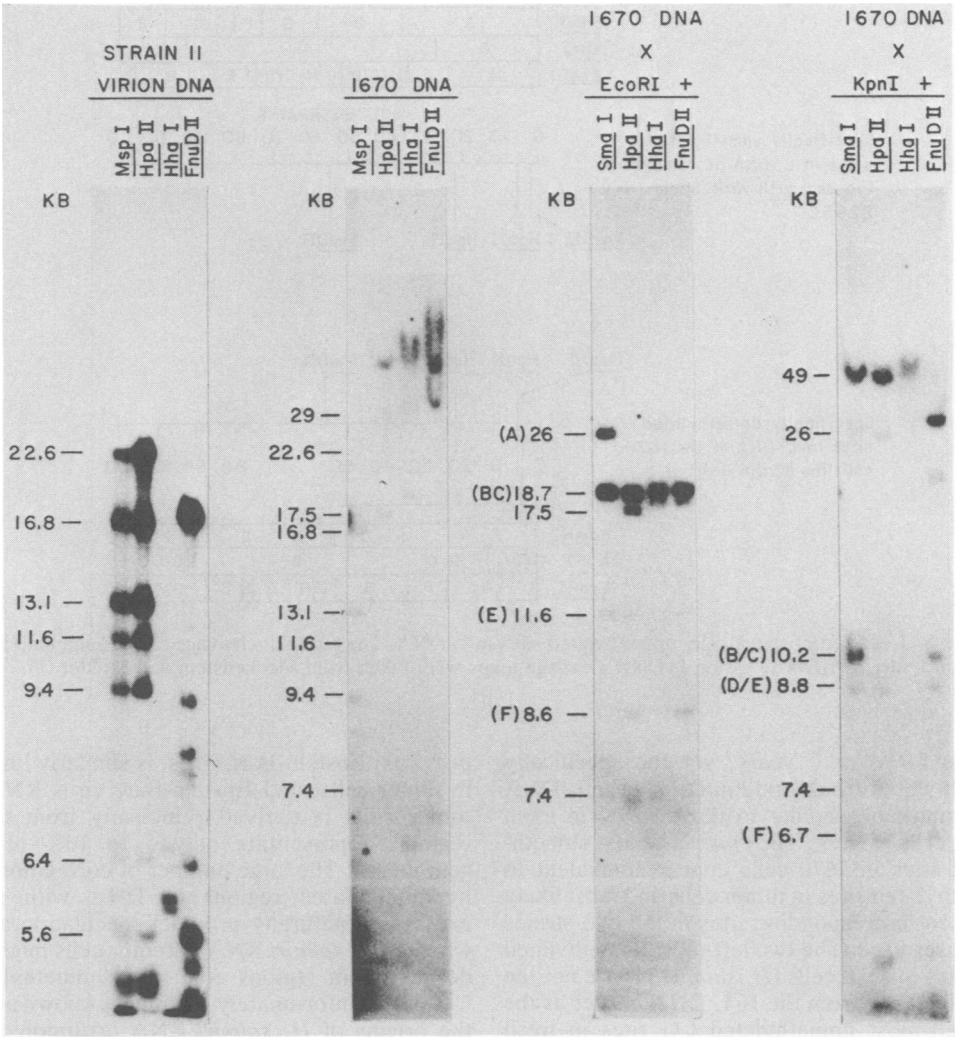

FIG. 5. Specifically unmethylated sites in L-DNA of 1670 cells. Digestion and analysis are as described in the legend to Fig. 3.

specifically unmethylated. From the band intensities of the autoradiograms it was estimated that these four sites were unmethylated in greater than 95% of the molecules, and the remaining 28 sites were methylated in greater than 90% of the molecules. The fact that the number and locations of the unmethylated sites appeared identical in the four tumor samples analyzed indicates that this is a reproducible, distinguishing characteristic. As with any restriction endonuclease mapping in the absence of DNA sequencing, it is not yet possible to determine whether what appears as one site may be actually two sites very closely separated, for example, by 20 nucleotides. Such a possibility seems statistically unlikely in this case, in which the 115-kb stretch of L-DNA contains only ~15 *Hpa*II sites, but DNA sequencing data will be needed to definitively answer this question.

For a DNA with 50% G+C, the sequence CCGG would represent approximately $0.25 \times 0.25 = 1/16 = 6.25\%$ of all CG sites. For a DNA with 36% G+C (L-DNA), the sequence CCGG would be approximately $0.72 \times 0.25 \times 0.25 \times 0.72 = 3.24\%$ of all CG sites. The CCGG and CGCG sites examined in this report thus represent approximately 6.5% of all CG sites in L-DNA. The analysis of the remaining CG sites (other than *Hha*I sites) is hampered by the lack of appropriate methylation-sensitive restriction endonucleases.

The 1670 cell line has been in continuous

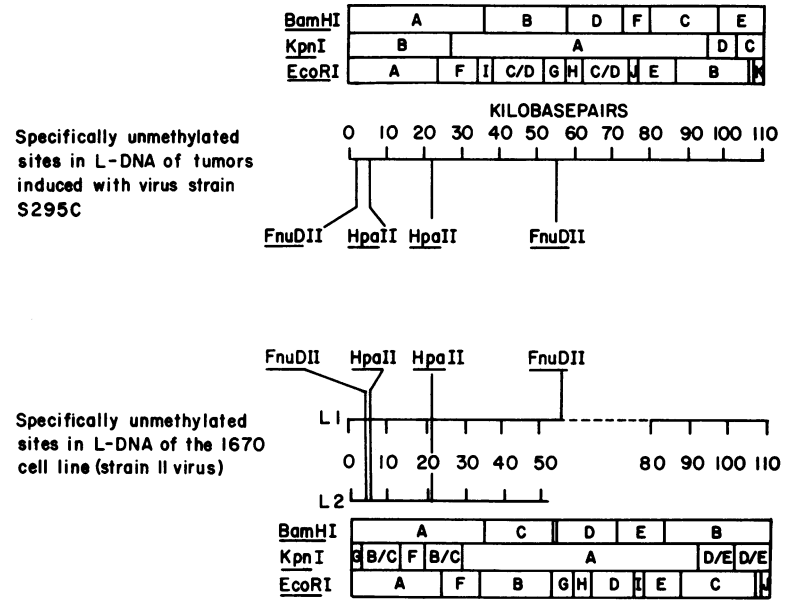

FIG. 6. Location of specifically unmethylated sites in L-DNA. The terminal cleavage site in each map is the first *Sma*I site in H-DNA. Virion L-DNA cleavage maps were taken from Fleckenstein and Mulder (9).

culture for over 7 years, yet the specifically unmethylated *Hpa*II and *Fnu*DII sites in L-DNA are remarkably similar to those found in fresh tumor cells (Fig. 6). The two right-most unmethylated sites in 1670 cells appear equivalent to unmethylated sites in tumor cells and most likely represent corresponding sites in the two strains of viruses used. The two left-most unmethylated CG sites in 1670 cells (*H. saimiri* 11) are not the same distance from the H-L DNA border as the two left-most unmethylated CG sites in fresh tumor cells (*H. saimiri* S295C). However, these unmethylated sites are located in a region in which little or no homology exists between these two virus strains. The fact that there are two specifically unmethylated sites in this region in each case underscores the similarity. The left-most 7 kb of L-DNA are particularly interesting because of the concentration of specifically unmethylated sites, the two groups of viruses which exhibit little or no homology in this region, and because two independently derived nononcogenic variants have deletions in this region (C. Mulder, J. M. Koomey, R. C. Desrosiers, L. A. Falk, M. D. Daniel, and B. Fleckenstein, unpublished data).

Extensive analysis by electron microscopy and by indirect immunofluorescence tests of tumor cells obtained directly from tumor-bearing animals has not yielded evidence for any virus particle production or production of late antigens (8, 13). Expression of the human herpesvirus, Epstein-Barr virus, is similarly limited in tumor cells, and Epstein-Barr virus RNA in tumor cells is derived principally from three regions, representing only 5 to 10% of the genome (4). The large number of correlations of hypomethylated regions of DNA with gene expression naturally prompts speculation as to whether *H. saimiri* RNA in tumor cells might be derived from regions containing unmethylated CG sites. Unfortunately, nothing is known about the origins of *H. saimiri* RNA in tumor cells; thus, future work will be directed toward determining which regions of *H. saimiri* DNA are being expressed in tumor cells.

Unmethylated foreign DNA such as bacterial plasmid DNA or the thymidine kinase gene, introduced into cultured cells via transfection, usually remains unmethylated after stable association with the cell (17, 18, 24). *H. saimiri*, as well as adenovirus (22), contrasts with this; these viral DNAs invariably are extensively methylated after stable association with the growth-transformed cell. If cellular methylase is methylating *H. saimiri* DNA, it remains to be explained how this particular methylation pattern arises. The unmethylated CG sites may be naturally refractory to methylation. Alternatively, these methylated and unmethylated sites could arise by chance in the millions of infected lymphocytes in the animal; if this led to expression of transformation-specific products and outgrowth of transformed cells, this particular

methylation pattern could be selected for and passed on to daughter cells. These possibilities of course do not exclude other explanations.

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