Haematology and Blood Transfusion Vol. 26
Modern Trends in Human Leukemia IV
Edited by Neth, Gallo, Graf, Mannweiler, Winkler

Episomal and Nonepisomal Herpesvirus DNA in Lymphoid Tumor Cell Lines

C. Kaschka-Dierich, I. Bauer, B. Fleckenstein, and R. C. Desrosiers

A. Introduction

Herpesviruses are able to persist for years or for life in certain cells of the host organism, for example, in neural tissue and cells of the lymphatic series. Little is known at the molecular level about the mode of herpesvirus persistence and the factors determining reactivation to secondary diseases, oncogenic transformation of appropriate target cells, elimination of virus-transformed cells by the immune system, and eventual outgrowth as a malignant tumor. The DNA of Epstein-Barr virus (EBV) is able to form circular molecules and to persist in human tumor tissues and lymphoblastoid tumor cell lines in the form of episomal viral genomes (Kaschka-Dierich et al. 1976; Lindahl et al. 1976). Some viral DNA, however, was found consistently to band with host cell DNA upon consecutive density centrifugations in CsCl gradients (Adams et al. 1973; Adams 1979). This has led to the conclusion that some EBV DNA is integrated into chromosomal DNA in transformed cells. There are, however, at least two problems with this interpretation. First, it is difficult to rule out all artifactual explanations, and secondly, methylation of viral DNA could cause a density shift toward the density of cellular DNA.

In an attempt to learn more about mechanisms of herpesvirus persistence and oncogenic transformation, we have concentrated our efforts on the highly oncogenic herpesviruses of New World primates, *Herpesvirus saimiri (H. saimiri)* and *Herpesvirus ateles (H. ateles)*. A lytic system for growth and the peculiar genome structure of these viruses offer advantages in the biochemical analyses of persisting viral DNA.

B. Structure of Episomal DNA

The complete genomes (M-genomes) of *H. saimiri* particles are linear duplex DNA molecules of slightly variable length, mostly with molecular weights between 96 and 110×10^6, which corresponds to about 136 to 166 kilo base pairs (KB). The M-genome contains a long, internal, unique segment of L-DNA [71.6 megadaltons (md)] with 36% guanine plus cytosine (G and C) and two terminal segments of repetitive H-DNA (71% G and C) which are variable in length. The repeat units of H-DNA have a 830,000 mol.wt.; they are strictly arranged in tandem, and the H-DNA sequences from both ends of the molecule are oriented in the same direction (Bornkamm et al. 1976; Fleckenstein 1979). *H. ateles* shares the characteristic features of this genome structure; L-DNA sequences in M-genomes of *H. ateles* contain 38% G and C; and the repetitive H-DNA termini have 75% G and C (Fleckenstein et al. 1978).

Autopsy materials from tumors induced by these viruses and lymphoid tumor cell lines contain substantial amounts of viral L- and H-DNA (Fleckenstein et al. 1977). Nonintegrated circular superhelical viral DNA could be isolated from *H. saimiri* and *H.ateles*-transformed cells by isopycnic and velocity centrifugation (Werner et al. 1977). Surprisingly, the size of viral episomal DNA in at least two of the cell lines exceeds that of virion DNA. The lymphoid tumor cell line No. 1670, derived from the infiltrated spleen of a cotton top marmoset *(Saguinus oedipus)*, carries circular viral DNA molecules of 131.5 md. Partial denaturation mapping in the electron microscope, computer alignment of the denaturation maps, and blot hybridizations with purified

radioactive virion DNA have been used to determine the structure of the viral episomes in tumor cell line No. 1670. Figure 1 summarizes our current understanding of the arrangement of L-DNA and H-DNA sequences in 1670 episomal DNA. The circular molecules contain two L-DNA regions and two H-regions. Both L-DNA segments are in the same orientation, and the shorter L-segment is a subset of the longer one. The longer L-DNA region appears to represent most of the L-region of virion M-DNA; however an internal piece of 12.5 md which encompasses the EcoRI D and H fragments of virion DNA is missing in the episome. The short L-region corresponds to about one half of virion L-DNA only. Thus, *H. saimiri* episomes of this cell line are a form of defective molecules.

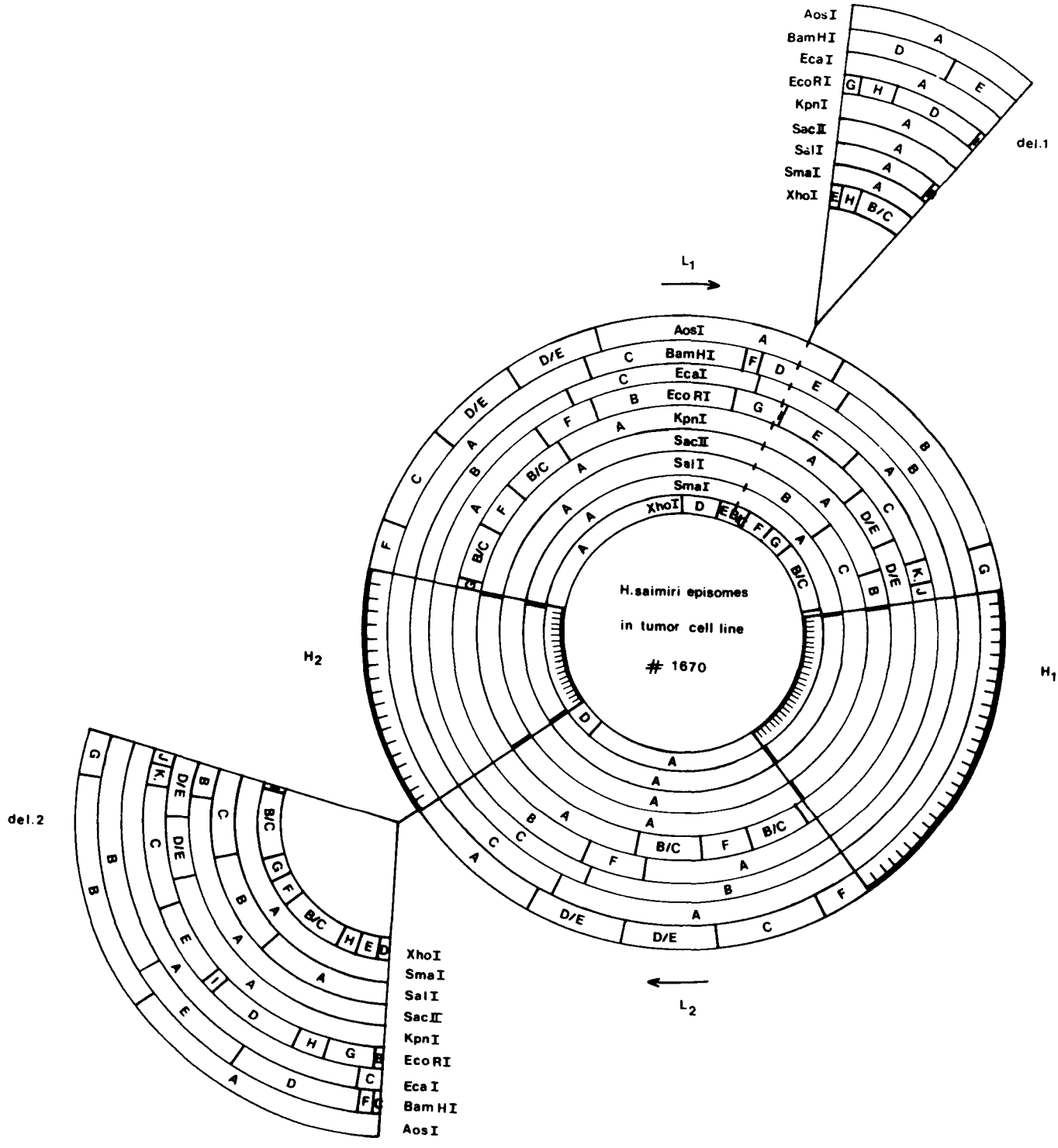

Fig. 1. Schematic representation of the arrangement of *H. saimiri* L- and H-DNA sequences in episomes from lymphoid tumor cell line No. 1670. Missing L-DNA segments are delineated as sectors outside the episome. The correlation between the cleavage maps of virion DNA and episomes was obtained by partial denaturation mapping in the electron microscope and by blot hybridizations

C. Nonepisomal Viral DNA

We have used DNA purified by ethidium bromide – cesium chloride gradients to separately analyze episomal and nonepisomal (linear) viral DNA by Southern transfer and hybridization to ^{32}P virion DNA. The EcoRI fragment D (9.8 md) and the EcoRI fragment H (4.0 md) cannot be detected as discrete bands in these blot hybridizations with 1670 DNA (Fig. 2). However, when EcoRI fragment D was isolated and used as a radioactive probe in solution hybridizations with total DNA from 1670 cells, the reassociation rate was accelerated in comparison to self hybridization in the presence of salmon sperm DNA (Fig. 3). This indicates that sequences of the EcoRI D fragment are indeed present in the 1670 cell DNA. These experiments must be extended using cloned DNA fragments and blot hybridization to determine the nature of the hybridizing sequences. It is possible that EcoRI D and H are absent as discrete fragments because they have been excised in an integration event, but further work is necessary to show this.

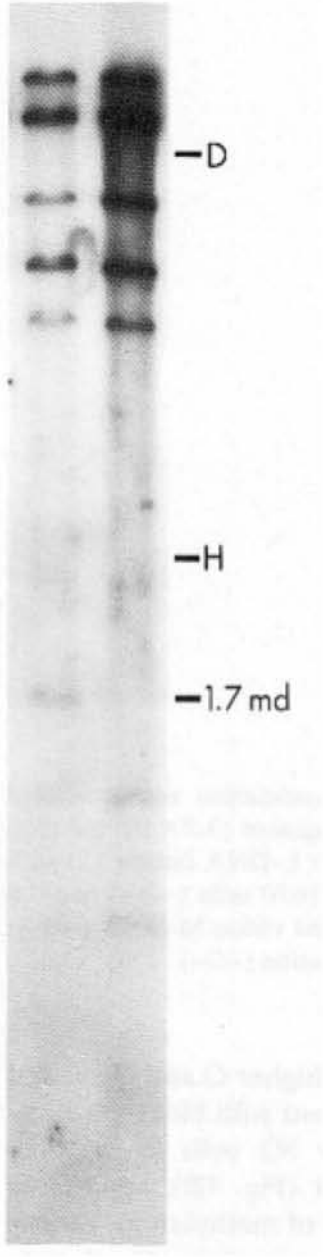

Fig. 2. Hybridization of DNA fragments from lymphoid tumor cell line 1670 to ^{32}P-labeled *H. saimiri* DNA. Nonepisomal DNA (the linear DNA fraction containing the bulk of cellular DNA) and episomal DNA was isolated from 1670 cells by centrifugation in ethidium bromide-cesium chloride gradients. The DNAs were cleaved with *endo* R. *Eco* RI and *Xma* I, transferred to nitrocellulose and hybridized to ^{32}P virion DNA. *Left lane,* nonepisomal DNA; *right lane,* episomal DNA

With some restriction endonucleases the linear DNA fraction described above yields discrete fragments not found in isolated episomal DNA. For example, digestion of isolated linear DNA of 1670 cells with endo R EcoRI and XmaI yields a 1.7 md fragment not observed with isolated episomal DNA (Fig. 2). Further work is needed to determine the nature of this viral nonepisomal DNA.

D. Methylation of Viral DNA

In the course of structural analyses of viral DNA in transformed cells by blot hybridizations we observed a striking resistance of intracellular viral DNA against the action of several restriction endonucleases. This has led us to conclude that viral H-DNA is extensively methylated in nonproducer cell lines (Desrosiers et al. 1979). Methylation of mammalian DNA has previously been found almost exclusively within the dinucleotide CG, forming 5-methylcytosine. Enzymes like endo R. Hpa II, Sac II, Sma I, which possess CG in their recognition site and are inhibited by methylation were found to cleave incompletely in the viral H-DNA from *H. saimiri*-transformed nonproducer cell lines No. 1670 and 70 N2, irrespective of DNA extraction procedure and excess of restriction enzyme. Enzymes without CG in their recognition site like endo R. Pst I, Pvu II, and Sac I cleave virion H-DNA and viral H-DNA from transformed cells identically. Msp I is an isoschizomer of Hpa II but unlike Hpa II cleaves whether or not the C of the CG dinucleotide is methylated. Viral H-DNA of 1670 cells, although resistant to cleavage by Hpa II, was cleaved to the same

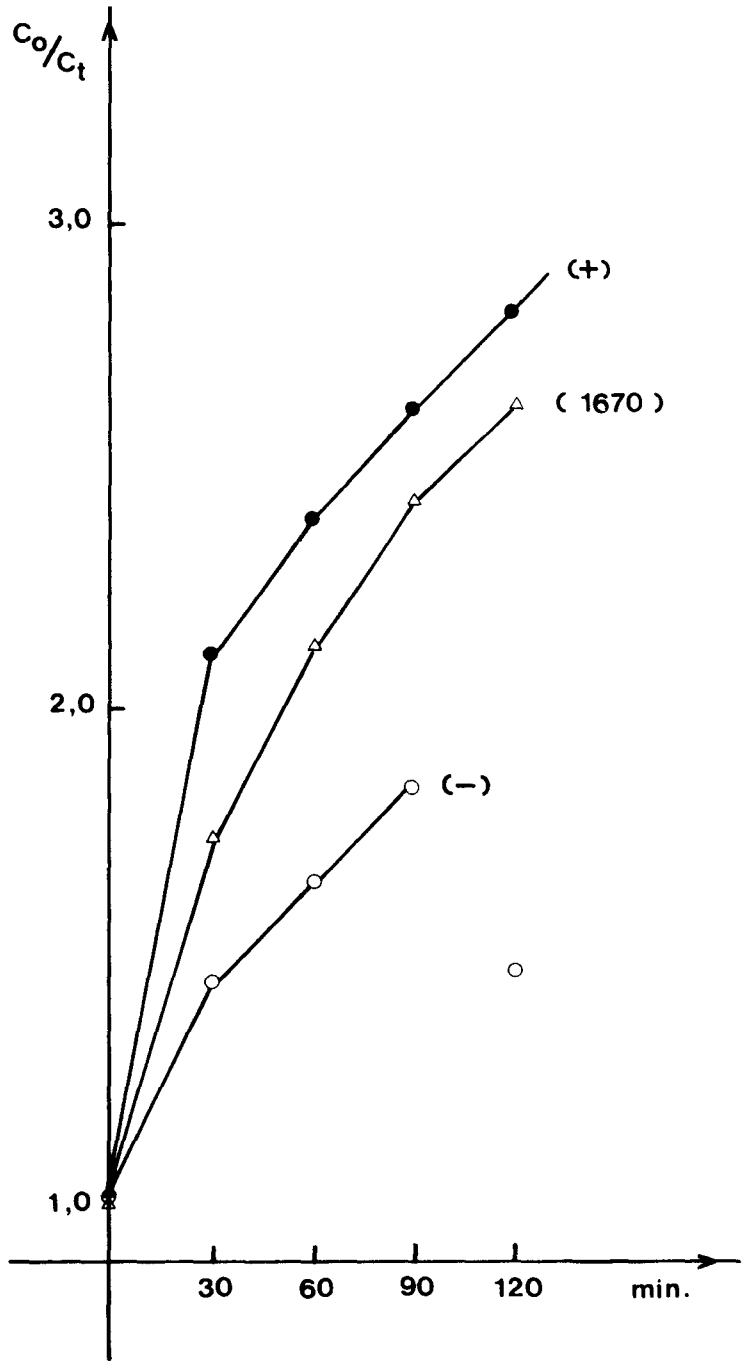

Fig. 3. Reassociation of ^{3}H-labeled *EcoRI* D fragment (3.7×10^4 cpm/μg) of *H. saimiri* L-DNA (strain 11) with DNA from 1670 cells (–△–) and 4.8 μg/ml purified virion M-DNA (–●–); self hybridization (–○–)

extent as virion DNA by Msp I. Using these restriction endonuclease criteria, we were not able to detect methylation of viral H-DNA in three transformed lymphoid cell lines producing virus. In addition, viral H-DNA in 1670 cells appeared somewhat more methylated than in 70 N2 cells.

A different approach for the detection of methylation in viral DNA was developed by measuring the buoyant density of viral DNA in transformed cells. The density of DNA in cesium chloride gradients is lowered by 5-methylation of cytosine (Kirk et al. 1967). As shown in Fig. 4 A), the viral DNA of 1670 cells is found at lower density than cellular DNA, in spite of a significantly higher G and C content in viral DNA. Consistent with blot hybridizations, DNA from 70 N2 cells shows only a slighter density shift (Fig. 4B), apparently due to a lower degree of methylation. Inhibitors of methylation may suppress the density shift of viral episomes in cesium chloride. After treating 1670 cells with 5'deoxy-5'-isobutyl adenosine (SIBA) or S-adenosyl homocystein (SAH) over 6 days, most viral DNA is found at about 1.710 g/ml which is the density to be expected from the average G and C content.

It remains to be seen if methylation of viral DNA plays a role in the lack of complete gene expression of *H. saimiri* in nonproducer lym-

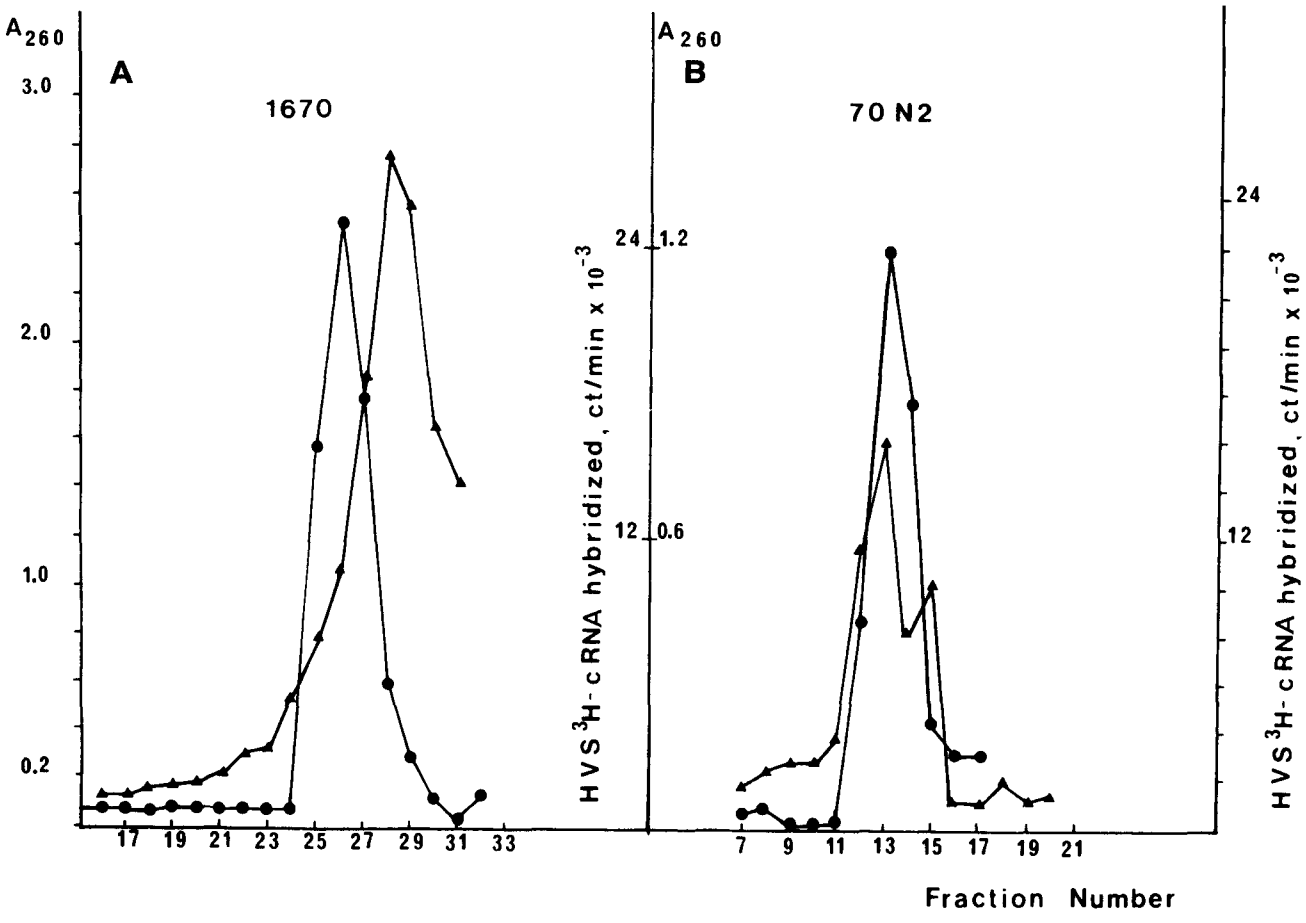

Fig. 4. Density centrifugation of DNA from cell lines No. 1670 (**A**) and 70 N2 (**B**) in cesium chloride. Cellular DNA (–●–); viral DNA monitored by ^{3}HcRNA hybridization on filters (–▲–)

phoid cell lines. The expression or lack of expression of hemoglobin and ovalbumin genes have also been linked to the state of their DNA methylation (McGhee and Ginder 1979; Mendel and Chambon 1979). Similar correlations were described for gene expression of adenovirus and mouse mammary tumor virus in transformed cells (Cohen 1980, Sutter and Doerfler 1980). If DNA methylation is indeed involved in gene expression, a multitude of virus-host interactions could be affected. DNA methylation could conceivably play a role in the control of gene expression in herpesvirus latency and oncogenic transformation.

E. Tumor Promotors and Methylation of Viral DNA in Transformed Cells

Since tumor promotors of the phorbol ester group like 12–0 tetradecanoyl-phorbol 13-acetate (TPA) are potent inducers of herpesvirus gene expression in tumor cell lines (zur Hausen et al. 1978), we recently asked whether these drugs may influence the state of methylation in *H. saimiri*-transformed lymphoid tumor cell lines. Preliminary experiments have shown that treatment of 1670 cells with TPA results in a significant increase of buoyant density for a substantial part of viral DNA (Fig. 5). Again, we do not know yet what the functional role of suppression of methylation may be in cells treated with a tumor promoting phorbol ester.

F. Summary

Tumor cell lines derived from *Herpesvirus saimiri (H. saimiri)*- and *Herpesvirus ateles (H. ateles)*-induced lymphomas of New World primates and rabbits contain multiple copies of viral genomes. Partial denaturation mapping and blot hybridizations of episomal DNA from lymphoid tumor cell line No. 1670 showed that a 12.5md-fragment is missing which represents the EcoRI D- and H-fragments of virion L-DNA. However, the missing piece can be demonstrated in total cellular DNA by reasso-

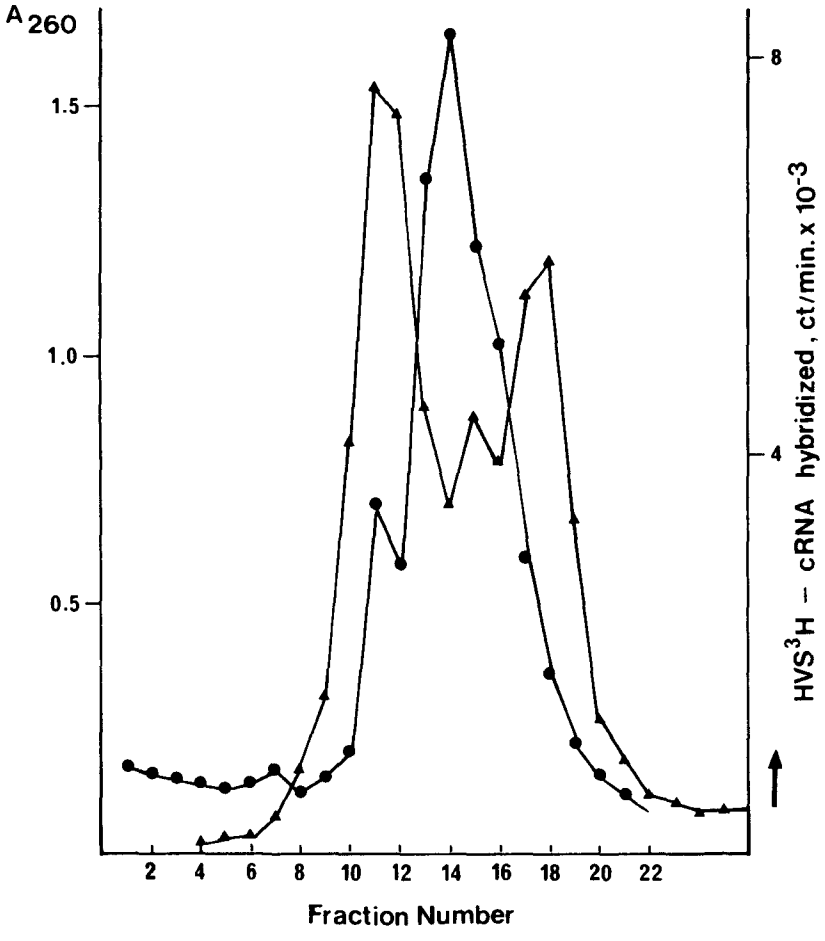

Fig. 5. Density centrifugation of DNA from cell line No. 1670, treated with 40 ng/ml TPA for 3 days. –●– cellular DNA –▲– viral DNA

ciation kinetics, possibly because it persists in integrated form. Both episomal and nonepisomal H-DNA are heavily methylated in a number of the lymphoid cell lines, and methylation may be reduced by conventional methylation inhibitors (S-adenosyl homocystein, SIBA) as well as by the tumor promoting phorbol ester TPA.

Acknowledgments

The work was supported by Stiftung Volkswagenwerk, Wilhelm-Sander-Stiftung, Deutsche Forschungsgemeinschaft, by contract No. 1 CP 81005 from the Virus Cancer Program of the National Cancer Institute, and by a fellowship from the Medical Foundation of Boston, Inc.

References

Adams A (1979). The state of the virus genome in transformed cells and its relationship to host cell DNA. Epstein MA, Achong BG (eds) In: The Epstein-Barr virus. Springer Verlag, Berlin Heidelberg New York, pp 155–183 – Adams A, Lindahl T, Klein G (1973). Linear association between cellular DNA and Epstein-Barr virus DNA in a human lymphoblastoid cell line. Proc Natl Acad Sci USA 70:2888–2892 – Bornkamm GW, Delius H, Fleckenstein B, Werner F-J, Mulder C (1976) The structure of Herpesvirus saimiri genomes: arrangement of heavy and light sequences within the M genome. J Virol 19:154–161 – Christman J, Price P, Pedrinan L, Acs G (1977) Correlation between hypomethylation of DNA and expression of globin genes in Friend Erythroleukemia cells. Eur J Biochem 81:53–61 – Cohen JC (1980) Methylation of milk-borne and genetically transmitted mouse mammary tumor virus proviral DNA. Cell 19:653–662 – Desrosiers RC, Mulder C, Fleckenstein B (1979) Methylation of Herpesvirus saimiri DNA in lymphoid tumor cell lines Proc Natl Acad Sci USA 76:3839–3843 – Fleckenstein B (1979) Oncogenic herpesviruses of non-human primates. Biochim Biophys Acta 560:301–342 – Fleckenstein B, Müller I, Werner F-J (1977) The presence of Herpesvirus saimiri genomes in virus-transformed cells. Int J Cancer 19:546–554 – Fleckenstein B, Bornkamm GW, Mulder C, Werner F-J, Daniel MD, Falk LA, Delius H (1978) Herpesvirus ateles DNA and its homology with Herpesvirus saimiri nucleic acid. J Virol 25:361–373 – Kaschka-Dierich C, Adams A, Lindahl T, Bornkamm GW, Bjursell G, Klein G, Giovanella BC, Singh S (1976) Intracellular forms of Epstein Barr virus DNA in human tumour cells in

vivo. Nature 260:302–306 – Kirk JTO (1967) Effect of methylation of cytosine residues on the buoyant density of DNA in cesium chloride solution. J Mol Biol 28:171–172 – Lindahl T, Adams A, Bjursell G, Bornkamm GW, Kaschka-Dierich C, Jehn U (1976) Covalently closed circular duplex DNA of Epstein-Barr virus in a human lymphoid cell line. J Mol Biol 102:511–530 – McGhee JD, Ginder GD (1979) Specific DNA methylation sites in the vicinity of the chicken beta-globin genes. Nature 280:419–420 – Mandel JL, Chambon P (1979) DNA Methylation: Organ specific variations in the methylation pattern within and around ovalbumin and other chicken genes. Nucleic Acids Res 7:2081–2103 – Sutter D, Doerfler W (1980) Methylation of integrated adenovirus type 12 DNA sequences in transformed cells is inversely correlated with viral gene expression. Proc Natl Acad Sci (USA) 77:253–256 – Werner F-J. Bornkamm GW, Fleckenstein B (1977) Episomal viral DNA in a Herpesvirus saimiri-transformed lymphoid cell line. J Virol 22:794–803 – Werner F-J, Desrosiers RC, Mulder C, Bornkamm GW, Fleckenstein B (1978) Physical mapping of viral episomes in Herpesvirus saimiri transformed lymphoid cells In: Stevens JG, Todaro GJ, Fox CF (eds) Persistent viruses. Academic Press, New York, pp 189–200 – zur Hausen H, O'Neill FJ, Freese UK Hecker E (1978) Persisting oncogenic herpesvirus induced by tumor promoter TPA. Nature 272:373-375