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Conservation and Progressive Methylation of Epstein-Barr Viral DNA Sequences in Transformed Cells

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The structure of intracellular viral DNA from a number of cell lines arising by clonal transformation of human lymphocytes in vitro with Epstein-Barr virus was analyzed. Intracellular viral DNAs were partially purified and digested with several restriction endonucleases, and the products of digestion were separated by electrophoresis in agarose gels. The viral fragments were detected by transferring the DNA from the gel to nitrocellulose sheets, hybridizing radiolabeled recombinant vectors carrying fragments of viral DNA to those transfers, and visualizing the hybrids by autoradiography. These analyses indicated that: (i) regions of repetitious viral DNA do undergo expansion and contraction although one size predominates; (ii) novel sequence arrangements appear in the intracellular viral DNA of different clones but are not found in clones analyzed serially and propagated extensively; (iii) the viral DNA is increasingly methylated upon cell propagation. We have not identified a transformed cell phenotype or a viral phenotype that segregates with the observed progressive methylation. We have not detected in Epstein-Barr viral plasmids analogs of the gross rearrangements of viral DNAs observed after lytic infections with high multiplicities of papova-, adeno-, or herpes simplex viruses.

Epstein-Barr virus (EBV) is a human herpesvirus which infects and transforms B-lymphocytes in vitro into continuously dividing lymphoblasts. Although transformation by EBV is efficient (10% or more of adult B-lymphocytes can be transformed, and one particle of EBV is sufficient to transform one cell [23]), lytic growth of EBV in human cells is a rare event. In fact, no cell type tested thus far supports efficient lytic growth of EBV (22). The major sources of virus are producer cell lines: EBV-transformed human or marmoset lymphoblastoid cell lines in which 1 to 5% of the cells release virus per cell generation. One such marmoset cell line, B95-8, has been used extensively as a source of transforming virus. The virus produced by B95-8 is potentially heterogeneous since B95-8 arose by cocultivation and has been in culture for many cell generations (13).

The viral DNA isolated from virions from cultures of B95-8 cells appears homogeneous, however (2). It is a linear duplex molecule having a molecular size of 115 megadaltons (Md) (16). Its termini contain multiple direct repeats of 0.37 Md which allow circularization of the viral DNA molecule in vitro after limited digestion with an exonuclease (7, 10). Its internal region consists of 20 Md of tandemly repeated DNA enclosed within 80 Md of unique DNA found in one orientation (5, 18). The internal repeat region maps 8% from one end of the DNA molecule and consists of 10 tandem copies of a 2.1-Md sequence (6, 8). The terminal and internal repeat regions share no sequence homology (6, 8).

The majority of intracellular viral DNA in nonproducer EBV-transformed cells is supercoiled, circular, and of approximately the same length as virion DNA (9, 11, 14, 28). Each cell contains multiple copies of this viral plasmid, and these copies can arise by preferential amplification of the viral DNA relative to cell DNA (24). Intracellular viral DNAs and virion DNA digested with restriction endonucleases yield cleavage products of similar sizes (10, 19, 21). These findings indicate that most of the intracellular viral DNA in EBV-transformed cell lines is equivalent to a circularized form of virion DNA.

No variation has been detected in the sequence arrangement of intracellular viral DNA isolated from cells transformed with a characterized stock of EBV. Rearrangements in the DNA may exist but may have not been observed for technical reasons. One such reason is that intracellular viral DNA cannot be purified to homogeneity and must be analyzed by using virion DNA as a probe. Previous studies of intracellular viral DNA have been limited by the

small amounts of virion DNA available and the difficulties of isolating pure, subgenomic fragments of virion DNA. If rearrangements were detected in intracellular viral DNA, the source of the variation would not be known because most cell lines studied have arisen by infection with an unknown number of virus particles of potentially heterogeneous virus pools.

We have circumvented these technical difficulties in two ways in order to search for rearrangements which may occur in the structure of intracellular EBV DNA during cell propagation. First, B95-8 DNA was sheared and cloned into lambda and *Escherichia coli* plasmid vectors so that small, pure fragments of viral DNA could be used as probes to analyze intracellular viral DNAs (G. Buell, D. Reisman, C. Kintner, G. Crouse, and B. Sugden, submitted for publication). Second, human lymphocytes were exposed to less than 0.05 particle of the B95-8 strain of EBV per cell and cloned in agarose so that the resulting transformants were likely to have arisen after infection with a single virus particle (23). Therefore, the viral DNA molecules in each of these clones of transformed cells are the progeny of a single B95-8 DNA molecule capable of inducing blast transformation. Samples of some cell lines were propagated in the presence of neutralizing antiserum to prevent reinfection by virions released infrequently from the transformed cells. In addition, the intracellular viral DNAs of two transformed cell clones were analyzed several times as the cells were propagated to gauge the stability of the viral DNAs as they replicated during cell growth.

These studies indicate, first, that during cell growth intracellular viral DNA undergoes variation in the number of units of repetition found in its two tracts of repeated DNA. Second, some sequence arrangements which appear to be novel to intracellular species can be detected infrequently. However, after 150 to 200 cell divisions the primary structure of the multiple copies of EBV plasmids is conserved, and the conserved species predominates in transformed cells. Third, as transformed cells are propagated, most of the intracellular viral DNA becomes resistant to cleavage by the endonucleases *Sal*I and *Hpa*II, but not to cleavage by *Msp*I. This finding indicates that some viral nucleotides are being methylated during cell growth.

MATERIALS AND METHODS

Cells, viruses and viral DNA. The virus used throughout this study was isolated from the supernatants of the B95-8 cell line (13) by methods described previously (23). The number of DNA-containing particles was determined by measuring the amount of viral DNA present by reassociation kinetics (24). The number of transforming units was titrated in a clonal transformation assay (23) using human peripheral lymphocytes as targets.

The in vitro EBV-transformed cell lines were generated by exposing peripheral lymphocytes from seronegative donors to 0.05 DNA-containing particle (0.005 to 0.002 transforming unit) per cell and cloning infected cells in agarose over a human fibroblast feeder layer (23). Colonies which arose were picked and established in culture as described previously (24). In some cases, a clone was divided into two portions soon after it was picked, and pooled human gamma globulin was added at 2 mg/ml to one of the portions to neutralize any virus released by the transformed cells. This concentration of human gamma globulin decreases by 300-fold the amount of virus detected by cocultivating lethally irradiated producer cells with seronegative lymphocytes (our unpublished data).

Intracellular viral DNA was isolated from 5×10^7 to 10^8 cells as described previously (10). The amount of viral DNA in a preparation of intracellular viral DNA was determined by reassociation kinetics. The ratio of viral DNA to total DNA and the assumption that the molecular weight of viral DNA is 10^8 and that of cell DNA is 4×10^{12} were used to calculate the average number of copies of viral DNA per cell (24).

Virion DNA was isolated as described previously (10) from virions released in cultures of B95-8.

Enzyme reactions, gel electrophoresis, and Southern transfer. *Hin*dIII, *Sal*I, and *Eco*RI were isolated as described previously (10). *Msp*I was purchased from New England Biolabs, and *Hpa*II came from Boehringer-Mannheim. Transformed cell DNA containing 300 ng of partially purified viral DNA was digested with *Hin*dIII, *Sal*I, *Eco*RI, *Msp*I, and *Hpa*II in a volume of 300 μl for 2 h at 37°C under the following conditions: *Hin*dIII, *Sal*I, *Msp*I, *Hpa*II—0.01 M Tris-hydrochloride (pH 7.7), 0.01 M $MgCl_2$, 0.05 M NaCl, and 100 μg of bovine serum albumin per ml; *Eco*RI—0.01 M Tris-hydrochloride (pH 7.4), 0.01 M $MgCl_2$, 0.2 M NaCl, and 100 μg of bovine serum albumin per ml. Restriction endonucleases were titrated on adenovirus type 2 DNA, and approximately 5- to 10-fold excess enzyme was used for the digestion of transformed cell DNA. In addition, 5 μg of adenovirus type 2 DNA was added to each digestion and then viewed by ethidium bromide fluorescence after gel electrophoresis to confirm that digestion was complete. Digestions were terminated by the addition of 0.05 M EDTA and then were extracted twice with phenol and twice with ether and finally were ethanol precipitated. Precipitated DNA was pelleted by centrifugation and suspended in 50 μl of 0.01 M Tris-hydrochloride (pH 7.4), 0.05 M NaCl, and 0.01 M EDTA. A 50-ng sample of digested intracellular viral DNA was applied to each slot of a 0.5% or 1.4% agarose gel and electrophoresed as described previously (10). DNA was transferred to nitrocellulose by the Southern technique (20) and prepared for hybridization as described previously (10).

Hybridization. The in vitro radiolabeling of DNA to a specific activity of 2×10^7 to 5×10^7 cpm/μg was carried out by nick translation using [α-^{32}P]dCTP or ATP and DNA polymerase I (10).

Approximately 0.5 μg of viral DNA was hybridized

to nitrocellulose for 24 h at 68°C under conditions as described previously (10). Nitrocellulose was washed extensively with 2× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and exposed to Kodak X-Omat X-ray film using intensifying screens at −70°C.

RESULTS

The clones of transformed cells studied were generated using varied conditions which might affect the structure of intracellular viral DNA. Clones were isolated after exposure of lymphocytes to 0.05 DNA-containing particle of EBV per cell. Some clones, as soon as they were isolated, were grown as parallel cultures in the presence or absence of neutralizing antisera to determine whether reinfection of cells by EBV released from a few cells within the clonal population contributed new structures to the pool of intracellular viral DNA. Two clones were propagated extensively and were sampled at different times to determine whether the length of time in culture affected the intracellular viral DNA. Major features of the clones studied are presented in Table 1.

Intracellular viral sequences complementary to the termini of virion DNA. The ends of virion DNA contain variable numbers of a direct repeat unit of 0.37 Md; as a result, numerous terminal fragments which vary in molecular mass by multiples of 0.37 Md are produced in restriction endonuclease digests of virion DNA (7, 10). These terminal fragments are not present in digests of intracellular DNA, which instead contain a new fragment (10). The molecular mass of this new fragment indicates that it is composed of the joined termini of virion DNA and usually contains multiple copies of the 0.37-Md terminal repeat.

A DNA fragment which includes virion DNA that extends from the rightmost *Hin*dIII site into the terminal repeats was isolated from the recombinant vector EB90-99 (Fig. 1B) (Buell et al., in preparation). This fragment was hybridized to digests of intracellular viral DNA to study the structure of the terminal repetition as it occurs intracellularly. When this *Hin*dIII terminal fragment was hybridized to *Hin*dIII/*Sal*I digests of intracellular viral DNA isolated from the clones listed in Table 1, one predominant fragment was detected in each digest. The results obtained for C5 and C5Ig, in which a 5.9-Md predominant fragment was detected, and for C6 and C6Ig, in which a 6.3-Md fragment was detected, are shown in Fig. 1A. There are several pieces of evidence that indicate that this fragment contains the joined termini of virion DNA, as follows. (i) The molecular mass for this fragment equals the sum of the unique terminal sequences of virion DNA (3 Md) plus 8 units of the terminal repetition for clones C5 and C5Ig, and it equals the sum of the unique terminal sequences plus nine units of the terminal repetition for clones C6 and C6Ig. (ii) The molecular mass for this fragment differs between clones by multiples of the unit length of the terminal repetition (0.37 Md); for example, the fragment is 5.9 Md for clone C5 and 6.3 Md for clone C6. (iii) When intracellular viral DNA was cleaved with other restriction endonucleases, for example, *Eco*RI, the molecular weight of the fragment changed, as would be predicted for the sum of the *Eco*RI terminal fragments (10). (iv) Finally, in intracellular viral DNA in which some sites

TABLE 1. *Major features of clones studied*

Clone	Approx cell division at which DNA was isolated[a]	Avg no. of copies of viral DNA per cell[b]	No. of cells releasing virus per 10^5 cells[c]
C1	50	170	<2
	200	3–5	<2
C2	50	NT[d]	NT
C3	50	5	3
C4	50		
	150	170	NT
C5[e]	30	130	<0.6[f]
C5Ig[e]	30	130	<0.6[f]
C6[e]	30	120	<1.4[f]
C6Ig[e]	30	120	<1.4[f]
C7	50	120	NT

[a] Estimation of the number of cell divisions that have taken place is based on the assumption that doubling times are constant over the life of the clone.

[b] Determined as described in the text, with the assumption that the molecular weight of human cell DNA is 4×10^{12}.

[c] This number was obtained by cocultivating γ-irradiated cells for 24 h with a 50-fold excess of seronegative leukocytes and then cloning cells in agarose. The values were derived from a standard curve using B95-8 cells, where for each two viral capsid antigen-positive cells cocultivated with leukocytes, one clone grew. The values with a "less than" sign indicate that no clones were observed and give the limit of detection for the experiment.

[d] NT, Not tested.

[e] Two clones were picked at approximately cell division 20, divided into two portions, and grown for another 10 cell divisions in the presence (C5Ig, C6Ig) and absence (C5, C6) of neutralizing antibodies.

[f] Virus release was measured at approximately cell generation 20.

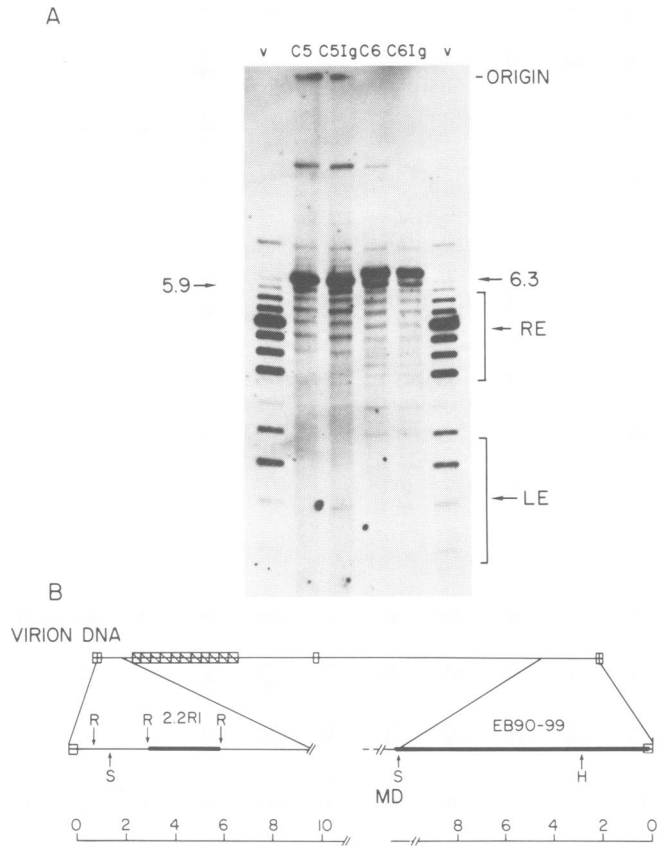

FIG. 1. *(A) Samples (50 ng) of virion DNA (slot v) and intracellular viral DNAs isolated from C5, C5Ig, C6, and C6Ig were digested with HindIII and SalI and were electrophoresed in a 0.5% agarose gel. The DNA was transferred from the gel to nitrocellulose and then hybridized with a ^{32}P-labeled 2.8 Md HindIII fragment isolated from EB90-99 [described below in (B)]. The molecular masses of the HindIII/SalI fragments detected by autoradiography are denoted in Md. RE refers to fragments in digests of virion DNA which contain the right terminus, and LE refers to those containing the left terminus. (B) Virion DNA is depicted with an expanded scale showing regions of interest. The hatched regions represent regions containing repetitious sequences. The central, single box represents a region homologous to the large internal repetition. R indicates sites in the DNA cleaved by the endonuclease EcoRI, S are sites cleaved by SalI, and H are sites cleaved by HindIII. The right half of the expanded scale shows the map location of the viral DNA insert contained in the recombinant vector EB90-99. The fragment used as a probe in (A) contains DNA from the rightmost HindIII site to the right terminal repeat. The left half of the expanded scale shows the map location of the 2.2-Md EcoRI fragment. This fragment was used as a probe in the experiment presented in Fig. 6.*

are resistant to cleavage by *Sal*I, the terminal fragment is occasionally joined to the *Sal*I/*Hin*dIII A fragment because the leftmost *Sal*I site of the DNA is not cleaved. Such a fragment was readily visible in the DNA of C5 and C5Ig, which contains more resistant sites than does that of C6 and C6Ig (Fig. 1A). When the *Hin*dIII terminal fragment from EB90-99 was hybridized to digests of DNA from five other clones which arose after exposure to high multiplicities of virus, three or more predominant fragments carrying the 0.37-Md repeat were detected in each digest (data not shown). That only one predominant fragment was detected by the *Hin*dIII terminal fragment in the viral DNA of the clones in Table 1 is consistent with each being transformed by a single virus particle.

In addition to the predominant fragments of 5.9 and 6.3 Md, the autoradiogram shown in Fig. 1A also reveals a "ladder" of fragments having weaker intensities than that of the predominating fragment. These minor species differ in molecular mass from the predominant species by multiples of 0.37 Md. The simplest interpretation of this finding is that these minor fragments represent joinings of the termini which contain

fewer or greater copies of the 0.37-Md repeat. Because these cells were transformed after exposure to 0.05 virus particle per cell and were cloned immediately after infection, it is likely that the intracellular viral DNA in each clone is the progeny of a single molecule. Therefore, the variation in the number of copies of the 0.37-Md repeat in the viral DNA is likely to have arisen after infection and within the transformed cell.

The pattern of fragments detected by the *Hin*dIII terminal fragment in Fig. 1A was identical in digests of DNA from clones propagated in the presence (C5Ig, C6Ig) and absence (C5, C6) of neutralizing antibodies (compare lanes C5Ig to C5 and C6 to C6Ig in Fig. 1A). Reinfection resulting from a few progeny of a transformed cell releasing virus between the 20th and 30th cell generations did not contribute to the rearrangements we detected in this region of intracellular viral DNA.

Finally, in addition to the minor fragments which vary by multiples of 0.37 Md from the predominant joint fragments, there are minor fragments which are the same size as some of the terminal fragments of virion DNA (compare lanes C6 and C6Ig with lane v of Fig. 1). The presence of these fragments indicates that linear viral DNA is present in some transformed cells.

Intracellular viral sequences complementary to internal, repetitious DNA. EBV virion DNA contains an internal region of tandemly repeated DNA which comprises about 20% of the molecule (6, 7, 18). The basic unit of this repeat is a sequence of about 2.1 Md that is cleaved once by *Bam*HI and is not cleaved by *Hin*dIII, *Sal*I, or *Eco*RI. There are approximately 10 repeats per virion molecule. However, a preparation of virion DNA will have molecules which contain fewer repeats. The internal repeats also share partial homology to other regions of the virion DNA (6; Buell et al., in preparation).

To analyze the region of intracellular DNA which contains the internal repeats of EBV DNA, we used a 2.1-Md *Bam*HI fragment of virion DNA which has been cloned into PBR322 (Buell et al., in preparation; map location shown in Fig. 2C). When hybridized to a *Hin*dIII/*Sal*I digest of virion DNA, this 2.1-Md *Bam* probe detected a fragment which contains the entire repeat region with flanking unique DNA (Fig. 2A, slot v at 30 Md). Smaller fragments with lower intensities were detected whose molecular weights are consistent with their containing fewer copies of the 2.1-Md repeat (denoted as Δ2.1 Md).

The data in Fig. 2A resulted from examining intracellular viral DNAs of clones which had been in culture for a short period of time (30 cell divisions) and were grown in the presence (C5Ig, C6Ig) or the absence (C5, C6) of neutralizing antibody for 10 generations. Barely detectable in the digests of intracellular viral DNA shown in Fig. 2A, and more apparent in longer exposures, are minor fragments which differ from the predominant bands by increments in 2.1 Md. Since the intracellular viral DNA is likely to be clonal in origin, the minor fragments represent rearrangements which arose after transformation. The intensity of these minor fragments was approximately the same in digests of the DNA of clones grown in the presence or absence of neutralizing antibodies, indicating that extracellular virus is not necessary for the generation of these rearrangements (compare slots C5 to C6Ig or C6 to C6Ig).

The presence of minor fragments, as detected by the *Bam*HI 2.1-Md fragment, was more apparent in most clones if they were examined after being in culture for greater than 30 cell generations. Figure 2B shows the results obtained when intracellular viral DNAs isolated from C1 at generations 50 and 200 and from C4 at generations 50 and 150 were digested with *Hin*dIII/*Eco*RI and hybridized with the *Bam*HI 2.1-Md fragment. In each case the *Hin*dIII/*Eco*RI digestion produced a predominant fragment of 25 Md (compared to 30 Md in a *Hin*dIII/*Sal*I digestion) and many minor fragments which differ by multiples of 2.1 Md. The predominant fragment of 25 Md appears to be maintained since it was present in C1 even after 200 generations in culture (Fig. 2B).

The arrangement of the unique regions of virion DNA in intracellular viral DNA. To detect variation in the sequence arrangement of the unique regions of intracellular EBV DNA, we used as overlapping set of Charon 4A λ vectors, each of which contains a fragment that represents about 10% of the virion DNA (Buell et al., in preparation). These recombinant phage DNAs were used as probes to compare endonuclease digestion products of intracellular viral DNAs with those of virion DNA. The results obtained from experiments using two such recombinants are presented here.

The recombinant phage designated EB38-47 contains a fragment of EBV DNA which maps to the region shown in Fig. 3B. Figure 3A shows the results obtained when this recombinant DNA was used to detect fragments of intracellular viral DNA resulting from its digestion with *Hin*dIII and *Sal*I. The most frequent or predominant fragments detected by EB38-47 in digests of intracellular DNA comigrated with predominant fragments found in digests of virion DNA.

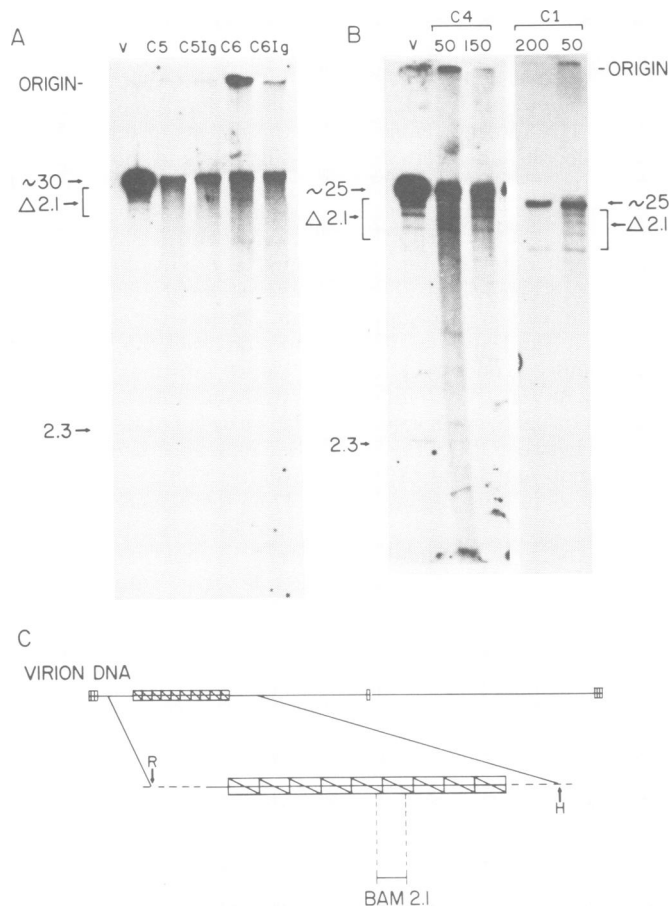

FIG. 2. *(A) Samples (50 ng) of virion DNA (slot v) and intracellular viral DNA isolated from C5, C5Ig, C6, and C6Ig were digested with HindIII and SalI and electrophoresed on a 0.5% agarose gel. The DNA was transferred from the gel to nitrocellulose and then hybridized with a ^{32}P-labeled BamHI 2.1-Md fragment [map location described in (C)]. The molecular masses of the HindIII/SalI fragments detected by autoradiography are denoted in Md. Δ2.1 refers to the set of fragments which vary in molecular mass by multiples of 2.1 Md from the predominant band. (B) Virion DNA (slot v) and intracellular viral DNA isolated from C4 at 50 and 150 generations and from C1 to 50 and 200 generations were cleaved with HindIII and EcoRI and electrophoresed in a 0.5% agarose gels. The hybridization and designations are the same as in (A). (C) Shown is a representation of virion DNA in which the hatched areas represent regions of repetitious DNA. The expanded scale shows the region of repeated DNA which is of interest. Each repeat unit within this region is cleaved once with BamHI, but it is not known whether each repeat unit is identical. Thus the 2.1-Md BamHI fragment of virion DNA cloned into PBR322 and used as a probe in Fig. 2A and B is known to map within the internal repeat region, but its exact location within the region is arbitrarily assigned.*

This result indicates that the majority of intracellular viral DNA homologous to EB38-42 is in the same arrangement as is virion DNA.

In addition to predominant fragments, the EB38-47 probe detects minor fragments in digests of intracellular viral DNA. Some of these fragments were exclusive to a particular clone (Fig. 3A, slot C3: 2.1, 5.1, and 5.5 Md), some were found in all clones and in virion DNA (Fig. 3A, 13.0-Md fragment), and some have molecular weights which are consistent with their arising from a cleavage site that is resistant to digestion with *SalI* (Fig. 3A, 9.2- and 8.8-Md fragments in slots C1, C4, and C2). The presence of resistant *SalI* sites in intracellular viral DNA will be discussed below.

The stability of the viral DNA in the region detected by EB38-47 during cell propagation was measured by analyzing the intracellular viral DNA isolated from C1 at both 50 and 200 generations and from C4 at both 50 and 150 generations after transformation. Figure 4 shows

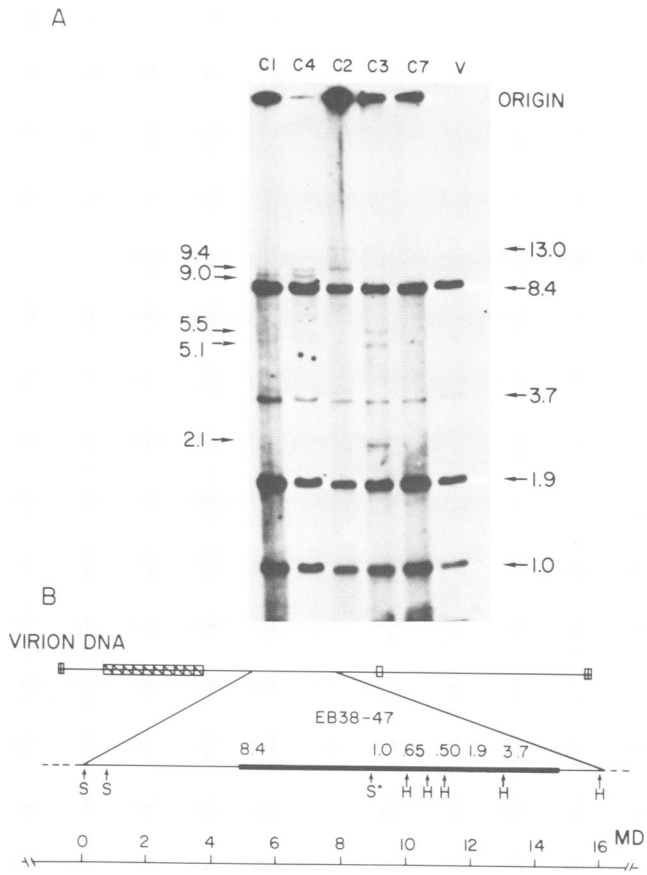

FIG. 3. *(A) Samples (50 ng) of virion DNA (slot v) and intracellular DNA isolated from C1, C4, C2, C3, and C7 at approximately 50 to 70 generations after transformation were cleaved with HindIII and SalI and electrophoresed in a 0.5% agarose gel. The DNA was transferred from the gel to nitrocellulose and then hybridized with a ^{32}P-labeled DNA from a Charon 4A-EBV recombinant designated EB38-47. The molecular masses of HindIII/SalI fragments detected by autoradiography are denoted in Md. (B) Shown is a representation of virion DNA where the hatched areas designate regions of repeated DNA. The expanded diagram shows the map location (heavy line) of the fragment of virion DNA contained in the Charon 4A-EBV recombinant EB38-47. Restriction endonuclease sites for SalI (S) and HindIII (H) are given below the line, and the molecular masses in Md of the HindIII/SalI double-digestion products are given above the line.*

the results obtained when *Hin*dIII/*Sal*I digests of these intracellular viral DNAs were hybridized to EB38-47. The major difference detected by EB38-47 between intracellular viral DNAs isolated from the two clones at different times was the presence of predominant fragments of 9.0 Md in the DNAs isolated after extended cell propagation. The intensity of these new fragments was greatest in the digests of DNA from C1 which had been in culture for 200 cell divisions. The molecular weight of these new fragments was consistent with their arising from the joining of adjacent *Sal*I fragments. Such joining would occur if the site recognized by *Sal*I were no longer sensitive to cleavage by that enzyme (see below). For example, if the *Sal*I site which is starred in Fig. 3B is not cleaved, then a 9.4-Md *Hin*dIII/*Sal* fragment would be detected (Fig. 4, slot C1-200), and a 1.0-Md fragment would disappear (Fig. 4, slot C1-200).

The recombinant phage designated EB83-93 has been found to contain a fragment of EBV virion DNA which maps to the region shown in Fig. 5B (Buell et al., in preparation). Some of the results obtained when intracellular viral DNA was probed with EB83-93 are shown in Fig. 5A. The results indicate that most of the predominant fragments detected in intracellular DNA comigrate with those detected in similar digest of virion DNA. Two additional fragments were detected in digests of intracellular DNA by EB83-93. One fragment of 20 Md was found only

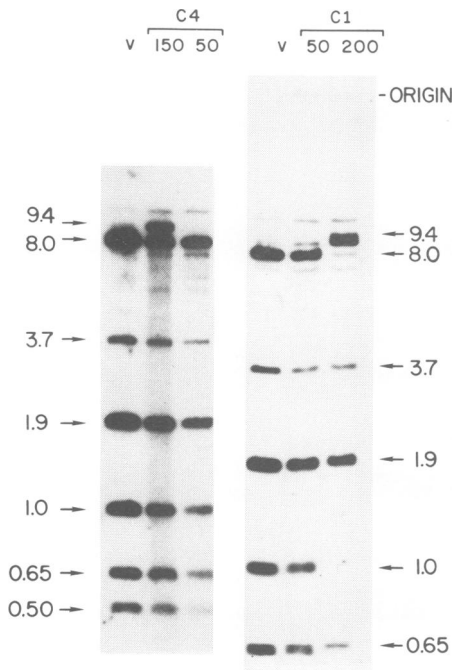

FIG. 4. *Samples (50 ng) of virion DNA (slot v) and of intracellular viral DNA from C4 isolated at 50 and 150 generations and from C1 isolated at 50 and 200 generations were digested with HindIII and SalI and electrophoresed on a 0.5% agarose gel. The DNA was transferred from the gel to nitrocellulose and then hybridized with ^{32}P-labeled DNA from the Charon 4A-EBV recombinant designated EB38-47 (see Fig. 3B). The molecular masses of the HindIII/SalI fragments detected by autoradiography are denoted in Md. The fragment designated 9.4 Md contains both a 9.0- and a 9.4-Md fragment which are not resolved in this experiment. They are resolved in the experiment shown in Fig. 3A.*

in the digest contained in slot C3 of Fig. 5A. The other fragment was found to varying extents in digests of several intracellular DNAs and has a molecular mass (13.5 Md) consistent with its arising when the *SalI* site starred in Fig. 5B is resistant to cleavage with *SalI*.

The stability of the region homologous to the virion DNA contained in EB83-93 was determined by hybridizing radiolabeled EB83-93 DNA to *Hin*dIII/*SalI* digests of intracellular viral DNA isolated from C4 at generations 50 and 150. The only difference in fragments detected by the EB83-93 probe in digests of virion and of C4 intracellular viral DNAs was the increase in intensity of the fragment of 13.5 Md in the digest of C4 DNA isolated at generation 150 (data not shown).

The comparison of fragments in digests of intracellular viral DNAs and virion DNA that are detected by other Charon 4A-EBV recombinant phages gave results similar to those obtained with EB38-47 and EB83-93. Those recombinant phages used constitute an overlapping set which together detect all fragments produced in an *Hin*dIII/*SalI* digest of virion DNA (Buell et al., in preparation). In all cases the predominant fragments detected in intracellular viral DNA comigrated with fragments found in similar digests of virion DNA.

***Hpa*II and *Msp*I digestion of intracellular DNAs.** When EB83-93 and EB38-47 were used to probe intracellular viral DNAs, fragments were detected whose molecular weights were consistent with their resulting from the joining of two fragments because of a failure to cleave *SalI* sites. In addition, this apparent resistance to *SalI* cleavage increased dramatically during cell propagation (Fig. 4, compare slots C1-50 with C1-200 and C4-50 with C4-150). The recognition sequence for *SalI* contains a CG sequence (1), and resistance to cleavage by *SalI* might result from the progressive methylation of the cytosine residues of intracellular viral DNA. Van der Ploeg and Flavell (26) found that the endonuclease *SalI* does not cleave DNA in which the C residue of the CG sequence is methylated. To test further the possibility that the viral DNAs were methylated, DNAs isolated from C1 after 50 and 200 cell divisions were digested with *Msp*I, which cleaves at CCGG whether methylated or not, or with *Hpa*II, which cleaves at CCGG only when its internal cytosine is not methylated (27). The digested DNAs were electrophoresed in a 1.4% agarose gel, transferred to nitrocellulose, and then hybridized with labeled fragments isolated from a recombinant phage. Figure 6 shows the results obtained when the 2.2-Md *Eco*RI fragment (map location shown in Fig. 1B) was used in this experiment to measure the extent of methylation of the sequence CCGG. The 2.2-Md *Eco*RI fragment contains a region of DNA which is heavily transcribed in nonproducer cell lines (17). Intracellular viral DNA homologous to the 2.2-Md *Eco*RI fragment isolated from the transformed cell clone, C1, at the 50th generation was cleaved with *Msp*I and *Hpa*II to the same extent. However, intracellular viral DNA isolated from C1 at the 200th generation was cleaved to a lesser extent with *Hpa*II than with *Msp*I. The results in Fig. 6 indicate that intracellular viral DNA is more methylated when isolated after 200 cell generations than when isolated after 50.

A similar experiment was performed in which intracellular viral DNAs were isolated from clone C1 at generations 50 and 200 and digested either with *Hpa*II or *Msp*I. This time the subset of viral sequences homologous to EB38-47 (Fig.

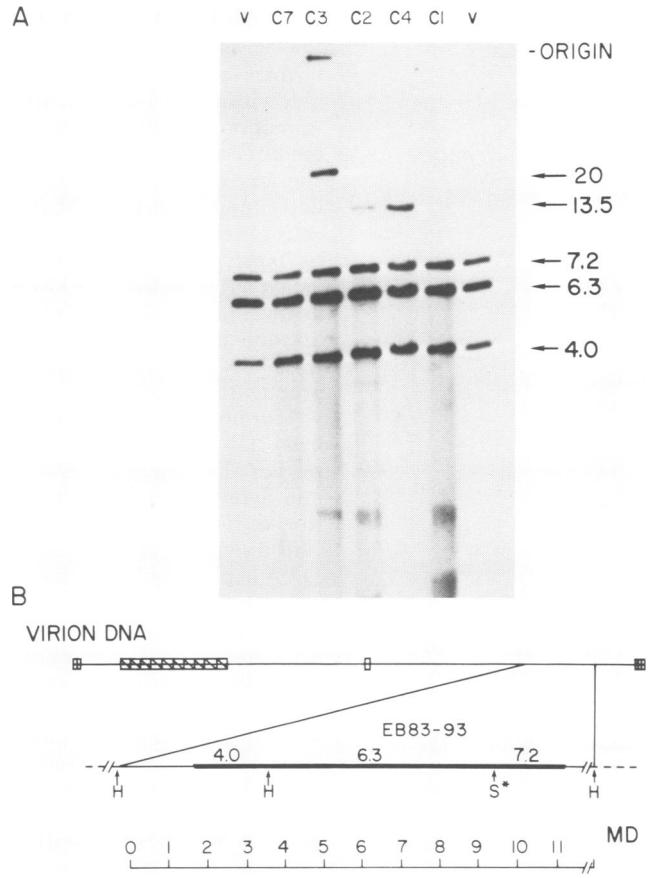

FIG. 5. *(A) Samples (50 ng) of virion DNA (slot v) and intracellular DNAs isolated from C7, C3, C2, C4, and C1 at approximately the 50th to 70th generation after transformation were cleaved with HindIII and SalI and electrophoresed in a 0.5% agarose gel. The DNA was transferred to nitrocellulose and then hybridized with ^{32}P-labeled DNA of the Charon 4A-EBV recombinant designated EB83-93. The molecular masses of the HindIII/SalI fragments detected by autoradiography are given in Md. (B) Shown is a representation of virion DNA in which the hatched areas designate regions of repeated DNA. The expanded diagram shows the map location (heavy line) of the fragment of virion DNA contained in the Charon 4A-EBV recombinant EB83-93. Restriction endonuclease sites for HindIII (H) and SalI (S) are given below the line, and the molecular masses of the fragments produced by digestion with HindIII and SalI are given above the line in Md.*

3B) were detected by hybridization. This region has not been found to be transcribed (15). Again, only the intracellular viral DNA isolated at the later passage of C1 was resistant to cleavage by *Hpa*II, but not by *Msp*I (data not shown), indicating that some sites of intracellular viral DNA are progressively methylated upon cell propagation.

DISCUSSION

The bulk of EBV DNA is maintained as multiple copies of plasmids in transformed lymphoblasts. Do the multiple copies of the EBV plasmid maintain the sequence arrangement found in virion DNA, or do they undergo sequence rearrangements which are favored during propagation of the transformed cell?

To answer this question we first cloned sheared, overlapping fragments of EBV virion DNA using Charon 4A or PBR322 as vectors. These pure fragments of viral DNA, which together span the genome, have complexities of about one-tenth of that of the whole molecule and have been used as probes to analyze each subregion of the intracellular viral DNA to which they are homologous. This analysis was applied to virion DNA to serve as a standard and to intracellular viral DNAs isolated from several transformed clones. The clones analyzed arose after exposure of cells to less than one viral particle per cell. Two clones were propagated in

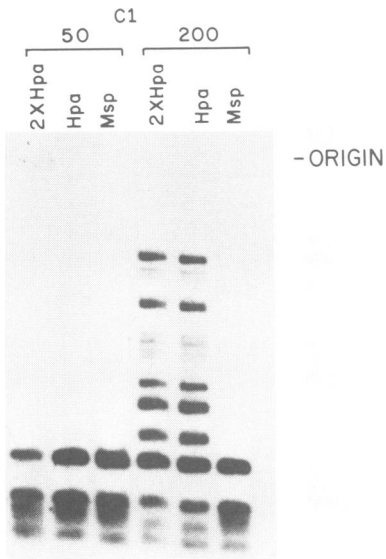

FIG. 6. *Intracellular viral DNAs were isolated from C1 at 50 or 200 generations after transformation and were cleaved with a 5-fold excess of MspI (Msp), with a 5-fold excess of HpaII (Hpa), or with a 10-fold excess of HpaII (2× Hpa). Digests were electrophoresed in a 1.4% agarose gel, transferred to nitrocellulose, and then hybridized with the ^{32}P-labeled EcoRI 2.2-Md fragment (map location shown in Fig. 1B). HpaII and MspI fragments hybridized to this probe were detected by autoradiography.*

the presence or absence of EBV-neutralizing antisera between cell generations 20 and 30.

The regions first analyzed in the different EBV-transformed cell lines were those containing repetitious sequences. There are two such regions of repeated DNA: a 0.37-Md direct terminal repeat and an internal 2.1-Md repeat. Both regions were analyzed using recombinant phage as probes, and in both cases the probe detected single, predominant fragments. Both probes also detected minor fragments which varied in molecular weight from the predominant species by multiples of the mass of their respective repeat units (Fig. 1 and 2).

The simplest interpretation of these two observations is that the predominant fragment detected in both cases represents the structure of the viral DNA soon after circularization, early during infection. The minor fragments which contain fewer or more repeats represent the structure of intracellular molecules which arose by deletion or insertion of repeat units, or both, during cell propagation. The generation of intracellular molecules which vary in the repeated regions does not seem to require infection by extracellular virus. This conclusion is based on the observations, first, that the EBV-transformed cells studied here released little detectable virus (see Table 1), and second, that addition of neutralizing antiserum to the cell culture medium at cell generation 20 had no effect on the amount of the variants detected. We have determined that the probability for transformed cells to release virus increases during the first 10 to 20 cell divisions after transformation (unpublished data), so that the low level of virus release for the studied clones (Table 1) measured at cell division 20 indicates the maximum free virus these dividing cells encountered during their propagation.

The nonrepeated regions of the intracellular viral DNA were analyzed using overlapping probes that spanned 90% of the unique DNA and detected all fragments generated by digestion with *Hin*dIII and *Sal*I. In general, *Hin*dIII/*Sal*I fragments of nonrepeated intracellular viral DNA comigrated with their homologs in digests of virion DNA. This result indicates that most of the intracellular viral DNA remains in the same sequence arrangement as virion DNA even after long periods (200 generations) of cell propagation. Minor fragments could be detected but were unique to a cell clone. These fragments apparently represent a sequence arrangement in intracellular viral DNA not found in EBV virion DNA. However, since they did not occur in cell lines in which we isolated the DNA at two different generation times, we have no estimate as to the stability of these novel arrangements. No such fragments were detected in the cell lines which were grown for greater than 100 generations.

The analysis of EBV plasmid DNA in a variety of transformed clones indicates that the predominant species found appear equivalent to circularized virion molecules. It appears that EBV has evolved such that its DNA is maintained stably as a plasmid in transformed lymphocytes.

The analysis of intracellular viral DNA identified some sites in the DNA that are resistant to cleavage by *Sal*I. After continued propagation, the number of resistant *Sal*I sites increased dramatically. Within our ability, we have excluded the possibility that the apparent resistance of these sites results from incomplete digestion. First, a 5- to 10-fold excess of each endonuclease was used for each digestion. Second, 5 μg of adenovirus type 2 DNA was included in each digestion of intracellular DNA and was found to be digested completely. Third, intracellular viral DNA of clone C4 was separately isolated four times, digested with *Sal*I and

*Hin*dIII, analyzed, and found to have the same resistant sites (data not shown). One possible explanation of these resistant sites is that the inability to cleave with *Sal*I is caused by methylation of cytosine residues and that methylation of viral DNA increases with time. This possibility is supported by the finding that *Sal*I does not cleave sites in which the C residue is methylated (26). It was further tested by isolating intracellular viral DNA from clone C1 both after 50 and after 200 generations and digesting it with *Msp*I or *Hpa*II. The results from this comparison indicate that some nucleotides of intracellular viral DNA are increasingly methylated upon propagation of the host cells in culture.

This progressive methylation is peculiar to the viral species. We have not detected a change in the relative sensitivity to digestion with *Hpa*II versus *Msp*I of either bulk cellular DNA or sequences homologous to a β-globin complementary DNA probe in those cells in which EBV DNA has become methylated upon cell propagation (our unpublished data). We do not know the biological significance of the observed methylation of viral DNA, and we have not yet identified a viral phenotype which segregates with the extent of methylation of viral DNA (3, 12, 25). We do not know whether transcribed regions are less methylated than nontranscribed regions of intracellular viral DNA. However, our observations do not support the notion that methylation alone dictates gene expression. Viral capsid antigens cannot be detected in the cells of a clone with unmethylated viral DNA, nor in its longer-propagated progeny which contains methylated viral DNA.

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