Intracellular State of Epstein–Barr Virus DNA in Producer Cell Lines

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SUMMARY

The physical state of the Epstein–Barr virus (EBV) DNA in three cell lines which spontaneously produce virus has been characterized. Circular EBV DNA molecules have been found in P3HR-1, B95-8 and M81 cells. The size of the intracellular M81 circular EBV DNA molecules is comparable with the linear virus genome isolated from virus particles but the circular P3HR-1 and B95-8 DNA molecules are shorter than the virion DNA. In addition to the circular form, some EBV DNA with physical properties indicative of integrated sequences was found in all three producer cell lines. There was no marked change in the amount of either the circular or integrated forms of EBV DNA when these producer cell lines were grown in the presence of phosphonoacetic acid to suppress the spontaneous virus production which occurs in a small percentage of the cells in untreated cultures.

INTRODUCTION

The majority of the latent Epstein–Barr virus (EBV) DNA molecules in transformed cells are not integrated and have a circular structure (for review, see Adams, 1979). However, some EBV DNA sequences apparently covalently bound to host cell DNA (Adams & Lindahl, 1975a) have been found in most cells. While the latter integrated virus DNA sequences are presumably replicated as a part of the host chromosome, the mechanism of replication of the circular, non-integrated, EBV genomes is not known.

Epstein–Barr virus production spontaneously occurs in a small percentage of the cells in growing cultures of producer cell lines while with non-producer cell lines there is no evidence of virus replication. Phosphonoacetic acid (PAA), an inhibitor of several herpes virus-induced DNA polymerases (Mao et al. 1975; Huang, 1975; Lee et al. 1976; Allen et al. 1977), blocks lytic EBV DNA synthesis (Nyormoi et al. 1976; Yajima et al. 1976), but the effect of the drug on the replication of the latent EBV genomes has not been fully characterized. The average number of EBV genome equivalents per cell decreases when virus producer cell lines like P3HR-1 (Yajima et al. 1976) and B95-8 (Summers & Klein, 1976) are grown in the presence of PAA. The reduction in EBV DNA load in such producer cell lines is probably due to the inhibition of lytic virus DNA replication that is restricted to only a small percentage of the cells in the untreated culture. With non-producer cell lines such as Raji, the number of latent EBV genome equivalents is not markedly altered by the PAA treatment (Summers & Klein, 1976; Yajima et al. 1976). The physical state of the latent EBV genomes in PAA-treated cells has not, to our knowledge, been reported.

In the present study, we have characterized the state of the EBV DNA of producer cell lines grown in the presence and absence of PAA. Virus producer cells were chosen for these experiments since any effect of the PAA treatment on lytic virus replication could be easily
monitored on the same culture used to prepare DNA for analysis. Moreover, the mechanisms controlling the expression of EBV DNA are not known and we were interested in observing any obvious difference between the intracellular state of the latent virus genomes in producer and in non-producer cell lines.

**METHODS**

**Cell lines.** Two marmoset lymphoid cell lines, B95-8 and M81, established respectively with virus from human lymphoid cell lines of infectious mononucleosis (Miller et al. 1972) and nasopharyngeal carcinoma (Desgranges et al. 1976) origin and the P3HR-1 human cell line, a clonal isolate of the Burkitt lymphoma derived Jijoye line (Hinuma et al. 1967), were employed. The lines were propagated at 37 °C in RPMI 1640 medium supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. For preparation of cell-free virus particles, cultures in medium with only 2% foetal bovine serum were incubated for 14 days at 37 °C. Phosphonoacetic acid treatment was at 80 μg/ml for a minimum of 8 days and two cell passages. The number of virus capsid antigen (VCA)-positive cells was determined by direct immunofluorescence with an IFTC conjugated serum (Klein et al. 1971).

**Characterization of EBV DNA.** The general experimental techniques used for the isolation and fractionation by gradient centrifugation of high mol. wt. DNA from lymphoid cells and the subsequent identification of EBV DNA sequences by nucleic acid hybridization have been described (Lindahl et al. 1976). In the present study, fractions from CsCl gradients of density 1.712 to 1.724 g/ml were pooled and analysed for free, non-integrated, EBV DNA forms, while fractions of density 1.699 to 1.711 g/ml were tested for the presence of integrated virus DNA sequences by repeated banding in CsCl (Adams et al. 1973). The presence of the covalently closed circular form of EBV DNA was indicated by a distinct peak of '100S' EBV hybridizing sequences on neutral glycerol velocity gradient centrifugation and/or virus DNA at the density expected for covalently closed circular DNA molecules in ethidium bromide-CsCl density gradients.

DNA sedimenting at 90 to 100S in neutral glycerol gradients or banding at heavy density in ethidium bromide-CsCl gradients was analysed for circular DNA molecules by electron microscopy. Prior to spreading, the covalently closed, supercoiled molecules were relaxed by the introduction of approximately two single-strand interruptions per molecule with a low dose of X-irradiation (Lindahl et al. 1976). Length measurements were made relative to the open circular form of bacteriophage PM2 DNA included on the same grid.

**RESULTS**

**Analysis of EBV DNA on neutral glycerol gradients**

The calculated average number of EBV genome equivalents per cell varies in producer cell lines depending on the level of virus production. However, when cultures are grown for 4 days in the presence of 80 μg/ml PAA, the number of VCA-positive cells is reduced by at least an order of magnitude and the number of EBV genome equivalents per cell decreases to a low value that remains constant on further growth in the presence of PAA (Nyormoi et al. 1976; Summers & Klein, 1976; Yajima et al. 1976). This residual number of EBV genome equivalents per cell probably approximates the number of latent virus genomes carried by the cells where virus production has not been spontaneously activated in the untreated cultures. Here, heavy density, high mol. wt. DNA isolated from three producer cell lines grown in the presence and absence of PAA has been analysed on neutral glycerol gradients.

Fig. 1 shows the nucleic acid hybridization profiles obtained with DNA isolated from the
Fig. 1. Glycerol gradient centrifugation of heavy density, high mol. wt. DNA from M81 cells. High mol. wt. DNA from an equal number of M81 cells grown in the presence or absence of PAA was isolated and fractionated by CsCl density gradient centrifugation. Fractions from the virus density region of several gradients were pooled, dialysed to remove CsCl and concentrated to a small volume. Bacteriophage T4 32P-DNA was added as a reference, and the DNA was centrifuged in 10 to 30% glycerol gradients containing 1 M-NaCl, 0.02 M-tris-HCl, 0.001 M-EDTA, (pH 8.0) in a Spinco SW27 rotor at 25000 rev/min and 20 °C for 220 min. Sedimentation is from right to left and the EBV DNA sequences were localized by nucleic acid hybridization of individual fraction with EBV 32P-cRNA.

M81 cell line. A small peak of EBV DNA sequences is observed on both gradients in fraction 9, where supercoiled circular DNA molecules of 100 × 10^6 mol. wt. would be expected to occur. Moreover, the amount of this fast sedimenting form of EBV DNA is similar in the PAA-treated and untreated samples. The major effect of the PAA treatment is on the size of the peak of EBV DNA sequences sedimenting slightly faster than the 61.8S phage T4 DNA marker. With DNA isolated from the untreated culture, this peak would comprise a mixture of linear virus genomes from those cells activated to virus production and the open circular form of EBV DNA. In contrast with the DNA sample prepared from cells grown in the presence of PAA, this peak is probably predominantly due to the open circular form of EBV DNA. In contrast with the DNA sample prepared from cells grown in the presence of PAA, this peak is probably predominantly due to the open circular form of EBV DNA because we routinely recover only 10 to 30% of the total intracellular circular EBV genomes in the covalently closed structure. Unfortunately, because most of the contaminating cellular DNA present in the heavy density DNA pool analysed also sediments at 50 to 70S, it is not possible to visualize the structure of the 60 to 65S forms of virus DNA by direct electron microscopic examination. The slowest sedimenting EBV DNA hybridizing material presumably consists of fragments of circular or linear molecules and has not been further characterized.

The sedimentation profiles of EBV DNA from B95-8 cells grown in the presence and absence of PAA were very similar to those of the M81 samples shown in Fig. 1. With B95-8 DNA the major peak on both gradients was at 62S while the faster sedimenting, presumably covalently closed circular form was calculated to have a sedimentation coefficient of 104S relative to the T4 DNA marker. As with M81 cells, PAA treatment reduced...
Fig. 2. Glycerol gradient centrifugation of heavy density high mol. wt. DNA from P3HR-1 cells. Experimental conditions were as described in Fig. 1. Only data from the control culture, containing 6% VCA-positive cells are shown.

the average number of EBV genome equivalents per cell to about 50 or one third that of the untreated culture.

The most profound difference between EBV DNA from PAA-treated and untreated cultures was seen with the P3HR-1 cells. In the sample prepared from cells grown in the absence of PAA, only a single, very large peak of EBV DNA was observed at 59S (Fig. 2). Thus, most of the EBV DNA in the virus-producing culture sediments at the same rate as the linear P3HR-1 genomes isolated from virus particles (Adams & Lindahl, 1975b; Pritchett et al. 1975). As in Fig. 1, there is a substantial amount of fast sedimenting material extending from the leading edge of the peak into the 100S region of the gradient, but no distinct separate peak indicating the presence of covalently closed circular molecules could be detected. Following PAA treatment, the total amount of EBV DNA in the P3HR-1 culture was reduced to only 2 to 5% of that of the untreated sample and it is thus not possible to present these hybridization data on the same scale as in Fig. 2. However, with several samples of DNA from PAA-treated P3HR-1 cells, a small peak (about twice the background level of EBV 32P-cRNA bound to control filters) could be seen in the 100S region in addition to a definite peak at 62S. The average sedimentation coefficient of the faster sedimenting form of P3HR-1 DNA, from three experiments, was 101S. In all experiments with DNA from PAA-treated P3HR-1 cultures, the main peak of EBV DNA sequences sedimented slightly faster than the 61.8S T4 DNA marker and was thus clearly different from the 59S form seen in Fig. 2 and in other experiments with DNA from untreated P3HR-1 cultures containing 5 to 10% VCA-positive cells.

Virus-producing cultures of P3HR-1 cells contain on average several hundred EBV genome equivalents per cell (Nonoyama & Pagano, 1971; zur Hausen et al. 1972; Pritchett et al. 1976) while in non-producing cultures of cells grown in the presence of PAA only about 10 EBV genome equivalents per cell are found (Tanaka et al. 1976; Yajima et al. 1976; present data). If the latter, latent EBV genomes only are circular, we estimate that, at most, 2% of the EBV DNA in our untreated P3HR-1 sample would have had a covalently closed circular structure and as such, a peak of 100S molecules could have been obscured by the high background of EBV DNA hybridizing material extending from the forward edge of the 59S peak on the neutral glycerol gradient. When heavy density, high mol. wt.
DNA from untreated P3HR-1 cells was instead analysed by ethidium bromide–CsCl density gradient centrifugation, a small peak of EBV DNA was indeed seen at the density of covalently closed circular DNA.

**Electron microscopic characterization of EBV DNA**

DNA sedimenting in the 90 to 110 region from the M81 and B95–8 gradients was screened for circular DNA molecules of approximately $100 \times 10^6$ mol. wt. using the electron microscope. Large circular DNA molecules were seen in all samples. The length of these molecules was measured and the results are presented in Fig. 3. The B95–8-derived DNA circles were smaller and had a somewhat broader size distribution than those from the M81 samples. However, there is no obvious difference between the size of the circles isolated from cells grown in the presence or absence of PAA with either cell line. The circular DNA molecules measured are all presumably EBV DNA because no such molecules were observed in a control experiment in which DNA isolated from uninfected cotton top marmoset lymphocytes and fractionated in an analogous fashion was examined in the electron microscope. Moreover, the finding of smaller circles in the B95–8 samples is consistent with the fact that the 883L human lymphoid cell line, which was the source of virus used in the establishment
Fig. 4. Glycerol gradient centrifugation of EBV DNA isolated from virus particles. Virus particles were concentrated 1000-fold by two cycles of centrifugation from 500 ml of a cell free culture supernatant from cells which had been incubated for 14 days at 34 °C. The virus concentrates were mixed with T4 3H-DNA and lysed by the addition of 1/2 vol. 3 % Sarcosyl, 0.075 M-tris-HCl and 0.025 M-EDTA, pH 9.0. The lysates were incubated for 2 h at 37 °C with 0.1 % pre-digested pronase. The DNA was then layered on a 10 to 30 % glycerol gradient as described in Fig. 1 and centrifuged for 300 min at 25,000 rev/min and 20 °C. Sedimentation is from right to left and the EBV DNA sequences were localized by nucleic acid hybridization of individual fractions with EBV 32P-cRNA. Data obtained with M81 DNA is shown. ●—●, Amount of cRNA hybridized; ○—○, T4 3H-DNA. P3HR-1 and B95-8 DNA analysed in parallel with the M81 sample gave sedimentation coefficients of 58.8S and 57.9S, respectively.

of the B95-8 line, contains atypically small circular EBV DNA molecules having a mol. wt. of only 94 x 10^6 (Adams et al. 1977).

The concentration of EBV DNA in the 100S region of the P3HR-1 glycerol gradients was less than a quarter of that in the B95-8 and M81 samples and it was therefore difficult to find circular DNA molecules in the electron microscope. The length of P3HR-1 circular EBV genomes was therefore measured on material having the density of covalently closed DNA molecules in ethidium bromide-CsCl. In agreement with the sedimentation rates of the circular EBV genomes (101 and 62S for P3HR-1 DNA versus 104 and 65S for M81 DNA) the circular EBV DNA molecules present in P3HR-1 cells are of a small size, having an estimated mol. wt. of 100 x 10^6.

Comparison of the circular EBV DNA molecules with virion DNA

Two of the three producer cell lines characterized here contain circular EBV DNA molecules which are estimated (from the electron microscopic data) to have mol. wt. of only 98 x 10^6 to 10^8. As such, the intracellular circular B95-8 and P3HR-1 DNA molecules are 5 to 10 % shorter than the 106 x 10^6 linear molecules isolated from virus particles (Pritchett et al. 1975). We have confirmed that DNA's isolated from B95-8 and P3HR-1 virus particles have coefficients of 58 ± 1S on co-sedimentation with 61.8S bacteriophage T4 DNA (see legend to Fig. 4). If the linear B95-8 and P3HR-1 molecules are synthesized using the circular forms as template, 5 x 10^6 to 10^7 daltons of DNA have been added. The linear virus DNA of B95-8 and P3HR-1 origin contain repeated DNA sequences (Rymo & Forsblom, 1978) and duplications of certain regions of the virus genome might have occurred during linearization.
Moreover, the terminal redundancy present in both B95-8 and P3HR-1 linear DNA molecules is estimated to be $3 \times 10^6$ daltons (Given & Kieff, 1978) and could also account for some of the difference seen. Restriction endonuclease analysis of the purified EBV DNA circles will be needed to resolve this question.

The sedimentation profile of EBV DNA isolated from M81 virus particles is presented in Fig. 4. Relative to the T4 marker, the main peak of EBV hybridizing DNA sequences is at 58-6S and M81 virion DNA is thus the same size as the other two isolates. However, in contrast to B95-8 and P3HR-1, the circular and linear forms of M81 DNA are of similar size.

Test for integrated EBV DNA sequences

Because of the large size of herpes virus DNA, it is difficult to demonstrate convincingly the integration of large segments of the virus genome. Moreover, as discussed elsewhere (Lindahl et al. 1978), the study of integrated EBV DNA sequences is even further complicated by the large excess of free, non-integrated, virus DNA molecules in most cells. The finding of some EBV DNA of an aberrant light density after most of the free, 1.718 g/ml density, EBV DNA molecules had been removed by repeated banding in CsCl (Adams et al. 1973) is, however, a good indication that some integrated virus sequences are present. DNA of density 1.699 to 1.711 g/ml from PAA-treated and untreated samples was re-banded in CsCl until the major peak of free EBV DNA molecules had been removed. The density distribution of the remaining virus DNA sequences was then determined by nucleic acid hybridization of individual fractions from the final CsCl gradient.

The results obtained with DNA from P3HR-1 cells grown in the presence and absence of PAA are shown in Fig. 5. Rebanding of the 1.699 to 1.711 g/ml density fractions of the first CsCl gradients of DNA from PAA-treated cells demonstrates that much of the EBV DNA in this pool has an aberrant light density (Fig. 5a). Less than half of the EBV DNA sequences are located to the left of the internal density marker, Klebsiella pneumoniae DNA (density 1.717 g/ml) where free virus genomes of density 1.718 g/ml would band. The total amount of hybridization in Fig. 5(a) is low and most of the approx. 10 EBV genomes present in PAA-treated P3HR-1 cells have a circular structure and were removed in the initial CsCl density gradient separation. The first rebanding of DNA from the control culture is shown in Fig. 5(b). First, it should be noted that while most of the EBV DNA is of virus density, the total amount of EBV DNA in the 1.699 to 1.711 g/ml density pool from control cells is 10 times that of the PAA-treated sample shown in Fig. 5(a). This result is not unexpected for there is always some tailing of the main peak of free EBV DNA molecules into the lighter density fractions and the amount of such DNA is dependent on the initial concentration of virus genomes. In the experiment shown in Fig. 5, there was about 25 times more EBV DNA in the control culture and it is therefore necessary to reband the DNA once to reduce the level of contaminating free virus genomes to that of the PAA-treated sample. Some EBV DNA sequences are seen tailing into the less dense region and on subsequent rebanding of this material a profile similar to that of the PAA-treated sample is obtained (Fig. 5c). The total amount of DNA in Fig. 5(a) and (c) is similar and thus the relative amount of EBV DNA of an aberrant density is equivalent in the PAA-treated and untreated samples. For P3HR-1 cells there could be approx. one integrated EBV genome per cell.

Similar results were obtained on rebanding DNA from B95-8 and M81 cells grown in the presence and absence of PAA. More than half of the small residual amount of EBV DNA present after removal of the free genomes was found to have a density of less than 1.715 g/ml in all four samples. Thus there is an indication that some integrated EBV DNA sequences of the type found in non-producer cell lines like Raji are also present in all three of the producer cell lines studied here. While it is not possible to quantify the exact
Fig. 5. Rebanding of P3HR-1 DNA by neutral CsCl density gradient centrifugation to test for the presence of integrated EBV DNA sequences. Fractions of density 1.699 to 1.711 g/ml were pooled from several CsCl density gradients, adjusted to contain 2 μg DNA/ml and supplemented with a density marker (Klebsiella pneumoniae 3H-DNA, ρ = 1.717 g/ml) and solid CsCl to a final density of 1.714 g/ml. The material was centrifuged to 20 ml amounts, overlayered with paraffin oil in a Spinco 60 Ti rotor at 33,000 rev/min for 60 h at 20°C. The gradients were collected as 0.4 ml fractions through a large hole in the bottom of the tube. The cellular DNA (ρ = 1.699 g/ml) was localized by A260 measurements, the DNA density marker by 3H radioactivity and the EBV DNA sequences by hybridization of filter-bound DNA with 32P-labelled EBV complementary RNA. O-- O, A260; O --- O, K. pneumoniae 3H-DNA; ▲ --- ▲, EBV 32P-cRNA hybridized. (a) First rebanding of DNA from PAA-treated culture; (b) first rebanding of DNA from control culture; (c) subsequent rebanding of DNA from control culture (fractions 21 to 26 in Fig. 5b and similar fractions from several parallel gradients).

The proportion of the virus DNA that is integrated, the total amount of less dense EBV DNA was similar in paired samples as shown in Fig. 5(b) and (c). Thus, there is no indication that PAA treatment may cause a perturbation of the relative proportions of circular FBV genomes and integrated virus DNA sequences.
DISCUSSION

The intracellular state of the latent EBV DNA in producer cell lines appears to be analogous to that previously found in non-producer cell lines of both malignant (Lindahl et al. 1976; Koliais et al. 1978) and non-malignant (Kaschka-Dierich et al. 1977) origin and in tumour biopsies (Kaschka-Dierich et al. 1976). Covalently closed circular EBV genomes were found in similar amounts irrespective of whether the producer cell lines were grown in the presence or absence of PAA. Moreover, EBV DNA with the properties expected of integrated sequences was present in all samples. Both the integrated as well as the free circular EBV genomes are apparently maintained by host, PAA insensitive, DNA polymerases and the virus induced, PAA sensitive, DNA polymerase used in productive EBV DNA synthesis is probably not expressed in the latently infected cells. An interesting difference between the material studied here and previously investigated cell lines is that the large majority of the different isolates of EBV non-producer cells contain EBV DNA circles of virus genome length while two of the three producer lines have EBV DNA circles of reduced size.

The major effect of the PAA treatment was on the amount of the slower sedimenting form(s) of EBV DNA. In the case of P3HR-1 cells, there was also a shift in the sedimentation constant of this material from 59S in the control to 62S in the PAA-treated sample. Circular DNA molecules with one or more single strand interruptions, sediment about 1.1 times faster than linear forms of the same mol. wt. (Helinski & Clewell, 1971). The failure to see a similar shift in the sedimentation rate of EBV DNA in the 60S region of the PAA-treated and untreated B95-8 and M81 samples is probably related to the fact that the latent circular virus genomes account for a significant proportion of the total EBV DNA in the untreated cultures of these lines. Marmoset cells would appear to release virus particles into the culture medium more efficiently than P3HR-1 cells thereby reducing the intracellular pool of linear EBV genomes.

In conclusion, the present data are consistent with a mechanism of PAA inhibiting a DNA polymerase involved in the synthesis of the linear 59S form of EBV DNA which is found in virions, while PAA has no effect on the properties of the latent virus DNA. Consequently, the isolation of circular EBV DNA molecules from virus producer cell lines for structural studies is considerably simplified.

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