Evidence for integrated EBV genomes in Raji cellular DNA

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ABSTRACT

Human lymphoid cell lines cannot be grown in long-term tissue culture, as a rule, unless the cells have been transformed by Epstein-Barr virus (EBV). The latent EBV DNA in established cell lines, is mainly present as free covalently closed circles but viral DNA sequences with properties of integrated DNA also seem to be present. We have extended the studies on the physical state of the EB viral DNA sequences in the cell line Raji which appear at a lower density than that for free EB viral DNA during fractionation on CsCl density gradients. In such material a novel EcoRI EBV DNA fragment is present, which hybridizes to viral sequences homologous to EcoRI A. This fragment is not present in free covalently closed circular EBV DNA. When this EcoRI fragment is further analysed with HindIII a smaller fragment than expected, which contains BamHI W sequences, is detected. The demonstration of this HindIII fragment and its characteristics as a joint, viral-host chromosome fragment will be discussed.

INTRODUCTION

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is regularly found in a latent state in tumor cells of two human malignant diseases, Burkitt lymphoma and anaplastic nasopharyngeal carcinoma (1). EBV-transformed B-cell lines can be established at high frequency from blood of infectious mononucleosis patients or from Burkitt lymphoma biopsies. Latently infected lymphocytes usually contain multiple copies of the viral genome (2,3). Herpesvirus latency differs in several respects from the transformed states induced by smaller DNA viruses in heterologous mammalian cells. Multiple complete copies of the herpesvirus genome are apparently present in the carrier cells both *in vivo* and in established cell lines grown *in vitro* (4,5,6,7). Most studies on the intracellular state of EBV DNA have been performed with the lymphoblastoid cell line Raji. Raji has been established in cell culture from a Burkitt lymphoma biopsy (8). Each cell contains an average of 50-60 EBV genome equivalents and expresses a virus-associated nuclear antigen, EBNA (2,9). EBNA is presently the only known virus-induced prodcut that is found in all EBV DNA carrying cells (10). The latent EBV DNA in Raji cells is mainly present

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as free covalently closed circular DNA molecules of viral genome length, but viral DNA sequences with properties of integrated species also seem to be present (11,12,13,14,15,16)EBV DNA (density 1.718 g/cm^3) and human cell DNA (density 1.700 g/cm^3) separate well in neutral caesium chloride (CsCl) gradients. Sequences homologous to viral DNA but banding at a lower density (14,17) have also been detected. Approximately 80% of the total EBV DNA in Raji cells can be recovered as free circular DNA molecules after fractionation on neutral CsCl-density gradients (11,12,13). Intracellular viral DNA banding at an anomalously low density in such gradients presumably represents integrated DNA. These EBV DNA sequences remain at the same low density after rebanding in CsCl gradients, but shift to a higher density if the molecular weight of the DNA is reduced by shear treatment. The EBV genome copies present in the in vitro converted sublines of Ramos have been demonstrated to be carried as integrated DNA sequences at the density 1.713 g/cm^3 by different fractionation methods (17). Those sublines contain only one to four EBV genome equivalents per cell and non-integrated viral DNA molecules appear to be absent. The previous data on integrated EBV DNA sequences in established human lymphoblastoid cell lines are all based on differential fractionation procedures and depend on a number of assumptions (17). In this work we have extended the studies on the physical state of the viral DNA sequences in Raji cellular DNA that band at an anomalously low density after fractionation on CsCl-density gradients. The DNA has been analysed by the restriction enzymes EcoRI, BamHI, and HindIII, followed by nucleic acid hybridization applying the Southern blotting technique (18). The study provides further evidence for the presence of a joint-fragment consisting of EBV DNA and cellular DNA sequences in Raji cells.

MATERIALS AND METHODS

Cell lines

The EBV-transformed human lymphoid cell line Raji (8) and the EBV negative cell line BJAB (19,20) were grown at 37°C as suspension cultures without agitation in RPMI 1640 medium supplemented with 8% total bovine serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. Actively growing cells, 5×10^5 to 1×10^6 cells/ml were used in all experiments.

CsCl-density gradient centrifugation

High-molecular cellular DNA was isolated from the Raji cells and fractionated in caesium chloride (CsCl) density gradients according to Lindahl et al. (11). The DNA with a density of 1.718 g/cm^3 was collected after the first set of CsCl-density gradients and used as a positive control in the experi-

ments (CCC DNA). The DNA banding between the densities 1.716 g/cm^3 and 1.700 g/cm^3 was pooled and further fractionated on two more density gradients. This purification was done in order to obtain a DNA preparation with no free covalently closed circular viral DNA molecules. After the third centrifugation the material to be investigated was collected as five fractions with densities between that of free viral DNA (1.718 g/cm^3) and cellular DNA (1.700 g/cm^3). Circular viral DNA from Raji cells was also prepared by the alkali method described by Griffin *et al.* (21).

Viral DNA and hybridization probes

Linear EBV DNA was prepared from virus particles released by the B95-8 cell line as described (22). The *EcoRI* and *BamHI* restriction enzyme fragments of EBV DNA have been cloned in pJC 79 and pBR 322 (23). These cloned fragments and the linear EBV DNA were radioactively labeled by nick translation using [α^{32}P]dCTP (24). The specific activity of the labeled DNA was 1-2 x 10^8 cpm/μg.

Gel electrophoresis of EBV DNA fragments

Purified cellular DNA from the five different fractions was incubated with *EcoRI*, *BamHI*, or *HindIII* under the conditions recommended by Boehringer and New England Biolabs, as modified for EBV DNA (21). Aliquots of 10 μg DNA were digested. Total unfractionated Raji and BJAB cellular DNA were cleaved under the same conditions, while only 1-2 μg of the CCC DNA was digested. *EcoRI* was used in an excess of 9 times, *BamHI* 3 times, and *HindIII* 3 times. They were all incubated at 37°C for 2 h, 4 h and 2 h respectively. The reactions were stopped by the addition of 0.25 volumes of 40% Ficoll - 0.1 M Tris-hydrochloride (pH 7.5) - 50 mM EDTA - 0.075% bromophenol blue - 0.1% SDS. The DNA fragments were separated on 0.35%, 0.5% or 0.8% agarose slabgels. Electrophoresis was performed for 18 h and 1.5 V/cm in E buffer (25). The separated DNA fragments were transferred to nitrocellulose (18) and hybridized to nick-translated ^{32}P-labeled (specific activity 1 x 10^8 to 2 x 10^8 cpm/ g) B95-8 EB viral DNA (26).

RESULTS

Isolation of material with properties of hybrid viral-cellular DNA

When the virus genome is large, as in the case of an EBV DNA molecule, the standard methods for preparation of high-molecular-weight DNA from mammalian cells yield shear-produced fragments of host chromosomes with an approximate molecular weight of 100 x 10^6 daltons, similar in size to the viral genome under study (27). In such cases, integrated viral DNA sequences should be found at intermediate densities on appropriate gradient centrifugation if

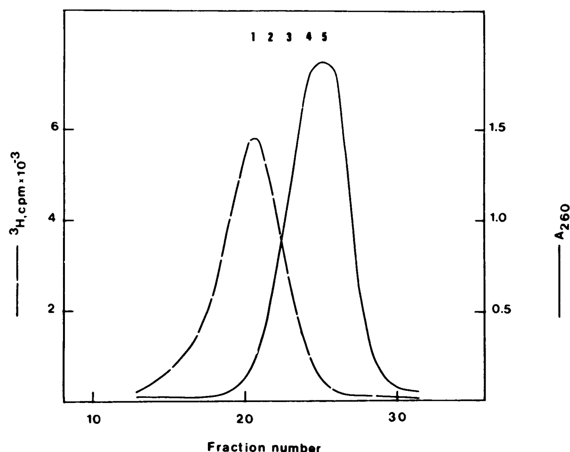

Fig. 1. CsCl-density gradient fractionation of human cellular DNA mixed with a trace amount of K. pneumoniae [^{3}H] DNA. The numbers 1 through 5 correspond to the five different DNA fractions collected after the third CsCl-density gradient centrifugation. Number 1 corresponds to the density of free EBV DNA (1.718 g/cm^3), number 5 to the cellular DNA density (1.700 g/cm^3), and numbers 2-4 contain DNA of densities intermediate to those two.

entire virus genomes are integrated. This is based on the assumption that the host genome is randomly fragmented into pieces comprised of varying proportions of viral and cellular DNA sequences (17).

In order to analyse the association between EBV DNA and the host genome in Raji cells, high molecular weight cellular DNA was fractionated on three consecutive CsCl-density gradients as described. This exhaustive fractionation of DNA to obtain material with densities less than 1.716 g/cm^3 had to be performed in order to isolate species free of covalently closed circular DNA molecules, which would otherwise complicate the analysis of integrated DNA sequences. The rebanded purified cellular DNA from the third set of gradients was collected as five different fractions. As can be seen in Fig. 1, fraction one contained DNA with the density of free EBV genomes while fraction five corresponded in density to cellular DNA. Fractions two to four contained DNA with densities intermediate to those of EBV DNA (1.718 g/cm^3) and cellular DNA (1.700 g/cm^3).

Characterization of the purified cellular DNA fractions

The five fractions isolated from Raji cells, containing cellular DNA with different densities were analysed with three restriction enzymes, EcoRI, BamHI, or HindIII. The covalently closed circular EBV DNA (CCC) obtained after banding the high molecular-weight DNA once in CsCl-density gradients and total un-

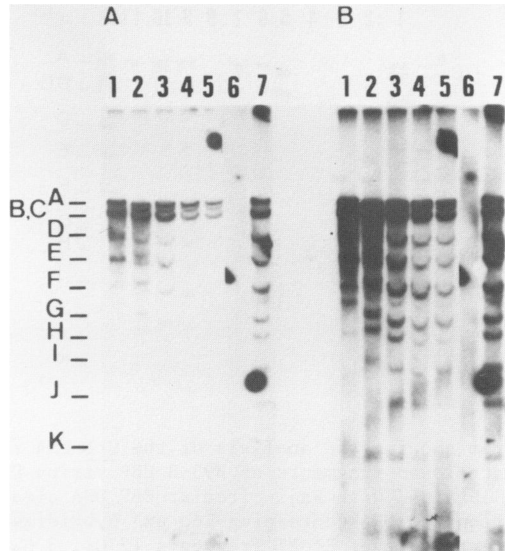

Fig. 2. Autoradiogram of EBV DNA sequences found in Raji cellular DNA following fractionation on CsCl. The EcoRI restricted DNA was separated by electrophoresis on a 0.5% agarose slab gel, transferred to nitrocellulose paper, and hybridized with labelled EB virion DNA. The letters (at left) correspond to bands obtained with Raji circular EBV DNA (37) cleaved with EcoRI. DNA material in lanes 1-5 correspond to the different CsCl-density gradient fractions. Lane 1 contains DNA with the density of free EBV DNA (1.718 g/cm^3), lane 5 is DNA with the density of cellular DNA (1.700 g/cm^3) while lanes 2-4 contain DNA of intermediate densities. Lane 6 contains the negative BJAB cellular DNA control and lane 7 is the positive control of unfractionated Raji cellular DNA. Panel A and B is the same autoradiogram exposed for different times to show that all the fragments are present in the different lanes. In order to be able to detect Eco exA in lane 7 the filter has to be exposed for a long time and then the actual area of the autoradiogram becomes too dark, because of the much higher concentrations of the normal A and B, C fragments relative to Eco exA (Panel B lane 7).

fractionated Raji cellular DNA were taken as positive controls, while BJAB cellular DNA was used as a negative control.

EcoRI cleavage pattern. Data on the analysis of cellular DNA fractions with regard to sequences complementary to EBV DNA are shown in Fig. 2. In all fractions EBV DNA sequences were detected with electrophoretic mobilities corresponding to fragments expected from cleaving virion DNA with EcoRI. The only difference observed was an extra DNA fragment that appeared between the EcoRI A and the B, C band in Raji cellular DNA (lanes 2-5). This new fragment (designated Eco exA), was not detected in fraction one (corresponding to density 1.718 g/cm^3), in covalently closed circular EBV DNA (see Fig. 4), or in total

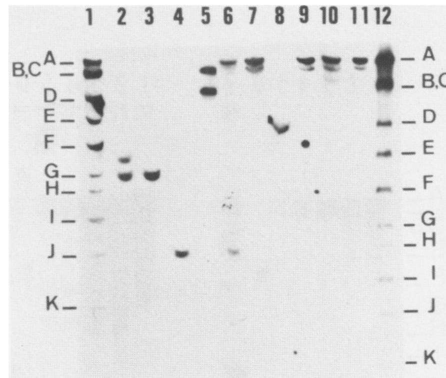

Fig. 3. Autoradiogram showing the analysis of the Eco exA fragment by hybridization against cloned fragments of B95-8 EBV virion DNA. The letters correspond to bands obtained with Raji circular EBV DNA cleaved with EcoRI. Lanes 2-11 are fractionated DNA containing Eco exA hybridized with different cloned EBV DNA fragments, either EcoRI fragments (2 G_1, 3 G_2, 4 J, 7 A, and 8 Dhet) or BamHI fragments (5 A, 6 C, 9 F, 10 H, and 11 W). EcoRI G_1 (2) hybridizes to F' and G_1. BamHI A (5) hybridizes to EcoRI C and D. EcoRI A (7), BamHI C(6), F (9), H (10), and W (11) hybridize to EcoRI A and Eco exA. BamHI C (lane 6) hybridizes also to EcoRI J because of overlapping sequences. The third band seen in lane 10 is probably due to homology between DS_L and the Not 1 repeats present in the probe and DS_R and the Pst 1 repeats present in Raji EBV DNA. The EcoRI digested DNA was either electrophorized on 0.5% agarose slab gel (lanes 1-6) or 0.35% (lanes 7-12). Lanes 1 and 12 show the positive controls unfractionated Raji cellular DNA and CCC DNA, respectively, hybridized with EB virion DNA.

unfractionated Raji DNA (lane 7). The new EcoRI fragment does not appear to be due to a partial digest of the DNA since it survives further digestion with the same enzyme, and it is not seen in total Raji DNA digested under the same conditions. Previous studies (11,12,13) indicated that EBV related sequences in total DNA from Raji cells consists (80%) of free EB viral DNA and approximately 20% which might be integrated in the host chromosome. Hence, for Raji cells, with 50-60 EBV genome equivalents, it is obvious that there is not only one copy of the EBV genome which is integrated into the host genome. This is supported by the observation that all the EcoRI virion DNA fragments are detected in the different fractions. The investigation of integrated sequences becomes complicated, because it has to be performed against the existence of all the normal restriction enzyme fragments.

Fractions containing DNA with the new EcoRI fragment (Eco exA) were pooled and analysed by hybridization, using different cloned EcoRI or BamHI EBV DNA fragments as probes. Such analyses showed (Fig. 3) that the Eco exA

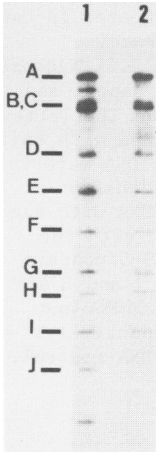

Fig. 4. A comparison of cellular DNA containing the Eco exA fragment and circular EBV DNA from Raji cells. Lane 1 shows EcoRI cleaved fractionated cellular DNA and lane 2 contains the digested circular EBV DNA. DNA fragments were separated on 0.35% agarose slab gel and hybridized to EB virion DNA. The letters correspond to bands obtained with Raji circular EBV DNA cleaved with EcoRI.

band contains DNA sequences homologous to the B95-8 virion EcoRI A DNA fragment (lane 7), while it did not hybridize to the EcoRI fragments such as Dhet, G_1, G_2, and J (lanes 2,3, and 4). Cloned B95-8 virion BamHI sub-fragments of EcoRI A were hybridized to Eco exA. The BamHI fragments C, F, H, and W (lanes 6,9,10, and 11) gave positive results. The BamHI A fragment did not hybridize to this new EcoRI fragment which is in agreement with the negative result obtained with the EcoRI Dhet fragment. The positive controls gave in all these experiments results expected from an analysis of EBV virion DNA with the corresponding cloned DNA fragments. The results show that a new EcoRI DNA fragment with sequences homologous to the B95-8 virion EcoRI A fragment is detected only in fractionated Raji cellular DNA of densities less than 1.718 g/cm^3. The molecular weight of this novel fragment was estimated to be 28×10^6 daltons or about 5×10^6 daltons less than EcoRI A.

The possibility of the existence of a different population of circular DNA, which is enriched for during repeated fractionations on CsCl-density gradients, has been investigated. Circular viral DNA from Raji cells was prepared by the relatively sensitive alkali method described by Griffin et al. (21). When the material obtained was analysed on an agarose slab gel after digesting the DNA with EcoRI the Exo exA band was not detected (Fig. 4), arguing against the presence of this species in circular EBV DNA molecules.

BamHI cleavage pattern. The five DNA fractions (above) were further analysed with BamHI. When B95-8 virion DNA was used as a probe all the BamHI DNA fragments were found to be present in the five fractions and no new bands were detected (Fig. 5). For further analyses, cloned EcoRI or BamHI B95-8 virion

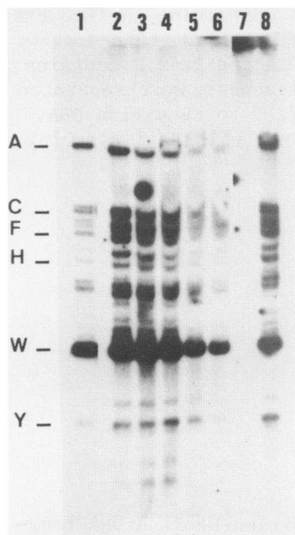

Fig. 5. Autoradiogram of EBV DNA sequences in CsCl fractionated Raji cellular DNA. The BamHI restricted DNA was electrophorized on 0.5% agarose slab gel, blotted onto nitrocellulose paper, and hybridized with labelled EB virion DNA. The letters correspond to some of the bands obtained with Raji circular EBV DNA cleaved with BamHI. DNA material 2-6 correspond to the different CsCl density gradient fractions. Lane 2 contains DNA with the density of free EBV DNA, lane 6 is DNA with the density of cellular DNA, while lanes 3-5 contain DNA of intermediate densities. Lanes 1 and 8 are the positive controls consisting of CCC DNA and unfractionated Raji cellular DNA, respectively. Lane 7 is the negative BJAB cellular DNA control.

DNA fragments were used as probes (Fig. 6). The EcoRI fragments A and G_2 (lanes 8 and 2) as well as BamHI fragments A, C, F, H, and W (lanes 3,4,5,6, and 7) gave a normal pattern. The whole EBV genome was found to be present in all five of the DNA fractions.

HIND III cleavage pattern. In order to investigate further the relationship between the novel Eco exA fragment (28 x 10^6 daltons) and EcoRI fragment A (33 x 10^6 daltons) fractionated DNA containing the Eco exA band was analysed with HindIII. Double digestion was also performed using HindIII and EcoRI in order to reduce the size of the unique fragment prior to further analysis. Covalently closed circular EBV DNA from Raji cells was similarly analysed (Fig. 7). When the material was cleaved with HindIII, and fragments identified using Bam HI W as a probe two fragments were detected. One migrating in a manner indistinguishable from the normal Hind III A fragment (size close to EcoRI A) and the other (designated Hind exA) was smaller than expected (lane 8). Its molecular weight was deduced to be approximately 7 x 10^6 daltons. This Hind exA fragment did not change in size when the material was further digested with EcoRI, whereas HindIII A was decreased to the size expected for a fragment consisting of BamHI C and Y and the W repeat fragments (lane 9). When CCC DNA was investigated under the same conditions the only fragment detected was the normal HindIII A fragment.

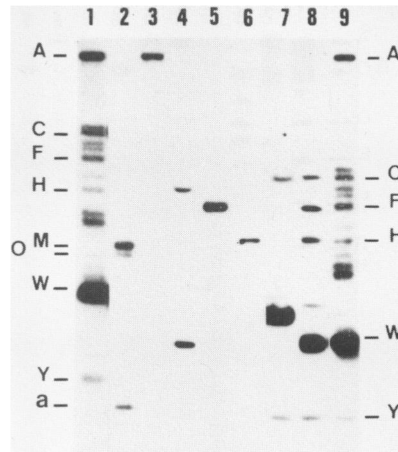

Fig. 6. Autoradiogram of BamHI restricted fractionated Raji cellular DNA containing the fragment Eco exA (detected in lanes 2-5 in Fig. 2). The letters correspond to some of the bands obtained with Raji circular EBV DNA cleaved with BamHI. The BamHI digested DNA was either electrophorized on 0.35% agarose slab gel (lanes 4, 8-9), 0.5% (lanes 1-3), or 0.8% (lanes 5-7). Lanes 1 and 9 show the positive control, CCC DNA, hybridized with EB virion DNA. Lanes 2-8 are fractionated DNA containing Eco exA hybridized with different cloned EBV DNA fragments, either EcoRI fragments (2 G_2 and 8 A) or BamHI fragments (3 A, 4 C, 5 F, 6 H, and 7 W). All the nick-translated fragments hybridize to the corresponding fragments according to the restriction enzyme maps (37). The extra bands detected in lane 4 and 7 are due to homologous sequences between the actual BamHI cloned fragments.

DISCUSSION

Cells transformed by tumour viruses consistently retain the viral DNA. It has been demonstrated for the smaller papovaviruses, adenoviruses, and provirus forms of RNA tumor viruses that the viral DNA is carried as linearly integrated sequences (28,29,30,31,32), or, in addition to integrated sequences, non integrated circular viral DNA molecules (33,34). In the case of oncogenic herpesviruses such as EBV, Mareks disease virus (MDV), and herpesvirus saimiri (HVS) it has been convincingly shown that virus transformed cells usually contain multiple copies of the viral genome. Work on this aspect of herpesvirus biochemistry has shown that EBV, HVS, and herpesvirus ateles (HVA) can occur as covalently closed circles in the transformed cell. Integration of herpesvirus DNA has not been seen in HVS infected cells (S. Schirm, personal communication). The same is true for Bovine papilloma virus transformed cells in which no integrated viral DNA sequences can be detected (35). This is in contrast to the smaller members of the papovavirus group where integration of viral DNA is a

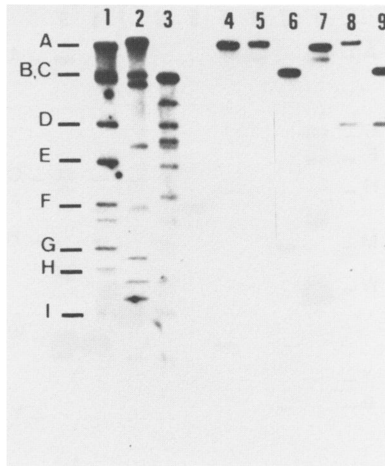

Fig. 7. Data showing the results obtained by digestion of fractionated Raji cellular DNA with HindIII, EcoRI and double digestion with the two enzymes. Lanes 1-3 show the CCC DNA hybridized with whole EBV DNA (1 EcoRI digested, 2 HindIII digested and 3 double digested). Lanes 4-6 are CCC DNA hybridized with BamHI W-fragment (4 EcoRI digested, 5 HindIII digested and 6 double digested). Lanes 7-9 show the Raji cellular DNA containing the Eco exA fragment hybridized with BamHI W (7 EcoRI digested, 8 HindIII digested and 9 double digested). DNA fragments were separated on 0.35% agarose slab gel and hybridized to BamHI W. CCC DNA was used as positive control and digested in the same way as the fractionated DNA and hybridized to BamHI W or EB virion DNA. The letters correspond to bands obtained with Raji circular EBV DNA cleaved with EcoRI.

normal phenomenon, although extrachromosomal species are also often observed. As earlier mentioned EBV DNA can be isolated from transformed cell lines as covalently closed circular molecules (11). In addition to such circles, EBV DNA with a hybrid density between that of cellular and free viral DNA was detected in such cells (19), indicating the existence of a covalent linkage between cellular and viral DNA.

In previous studies on integrated virus DNA in transformed cell lines, with a number of different viruses, "true" integration has been demonstrated by the identification of so-called joint-fragments, i.e. restriction enzyme generated DNA fragments consisting of both virus and cellular DNA. It is obvious from the results obtained on Raji cellular DNA that there is more than one copy of the EBV genome which might be inserted in the host DNA, since only about 80% of the total EBV DNA in these cells appear as free covalently closed circles (11,12,13). If the remaning 20% of the 60 EBV genome equivalents present in Raji cells is found at lower densities (<1.718 g/cm^3) it means that

approximately 12 copies of the complete viral DNA are associated with the host genome. This is supported by the observation that all viral DNA sequences are detected in the five DNA fractions analysed here and that they can be transferred to material cosedimenting with viral DNA at a density of 1.718 g/cm^3 by reducing the size of the DNA either by shearing (19) or restriction enzyme digestion (manuscript in preparation). In our experiments, in addition to the new DNA fragment which is detected after digestion with EcoRI, all the bands expected from cleaving virion DNA can be detected, including the Dend-fragment which is normally found only in circular DNA. These observations suggest that EBV DNA may be tandemly integrated and that the ends of the linear virion DNA molecule are not involved in any recombination event.

The new EcoRI fragment (Eco exA) which is detected in total DNA from Raji cells has DNA sequences homologous to EcoRI fragment A and the corresponding BamHI fragments. When DNA material containing Eco exA is further analysed with HindIII, two fragments were obtained, one correspondning to the expected HindIII A fragment and the other smaller than the fragment obtained after cleaving the corresponding virion DNA; the latter containing homologies to BamHI W. It is noteworthy that HindIII A has an electrophoretic mobility close to EcoRI A and does not migrate faster as it should if one compares the size of the two fragments (23,36). The HindIII digested DNA was further cleaved with EcoRI with no reduction in size of the small fragment; whereas the normal A fragment was decreased to the size expected. One explanation for these data is that there is both a new HindIII and EcoRI site in the Eco exA fragment which are located in non-viral DNA, the EcoRI site being located outside the HindIII site. The observation that the Eco exA fragment is homologous to BamHI F and H, suggest that it contains the normal HindIII cleavage site located in BamHI Y in B95-8 DNA. Furthermore, the Eco exA fragment was found to give a positive hybridization with BamHI C but not with EcoRI J. This positive reaction with BamHI C can be explained as being due to the homology between BamHI C and W. This is further supported by the observation that the size of the HindIII fragment, is estimated to be only 7 x 10^6 daltons. Thus, besides BamHI W (2 x 10^6 daltons) and half of BamHI Y (1.2 x 10^6 daltons), it can contain only a small amount of cellular DNA. Taken all together, the data indicate that the extra HindIII fragment could originate from integrated sequences and consist of half of the BamHI Y, 1-3 repeat (BamHI W) fragments, and cellular DNA (see Fig. 8).

In the present work only one joint fragment is detected when Raji cellular DNA is investigated with the restriction enzymes EcoRI or HindIII. A possible

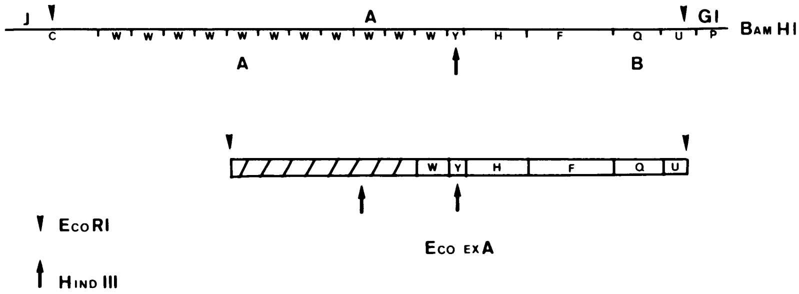

Fig. 8. A portion of the restriction endonuclease cleavage map of circular EBV DNA from Raji cells with the relative positions of the BamHI, EcoRI and HindIII fragments indicated (top). At the bottom of the figure is a schematic representation of the Eco exA fragment. The striped area corresponds to the predicted non-viral DNA with the new restriction enzyme cleavage sites.

explanation to this is that the other joint contains only small amounts of viral DNA sequences which are difficult to detect, because of the presence of the normal EBV DNA fragments. The size of this other joint fragment might also be comparable to that of a normal EBV DNA fragment containing the same viral DNA sequences. Our results emphasize the difficulty in analysing the physical state of EBV DNA with respect to integration of viral DNA into the genome of human transformed lymphocytes. The presence of a new EcoRI fragment (which now can be further analysed) and the observation that all the viral DNA present at intermediate densities can be transferred to a higher density after restriction enzyme digestion (manuscript in preparation) provide evidence for the fact that a significant proportion (10-12 copies) of the EBV genome present in Raji cells is linearly integrated in the host genome. The fact that a large number of joint fragments has not been found indicate that EBV DNA is integrated in tandem. Furthermore it is noteworthy that the linkage between viral and cellular DNA, as suggested in this paper, is found in a region of the viral genome which contains homologous sequences to cellular DNA. The significance of integrated EBV DNA in relation to the transformed state of the cells is unclear.

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