Linear Association Between Cellular DNA and Epstein–Barr Virus DNA in a Human Lymphoblastoid Cell Line
(provirus/Raji cells/density gradient centrifugation/nucleic-acid hybridization)

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ABSTRACT High-molecular-weight DNA from cell line Raji (derived from Burkitt’s lymphoma), which contains 50–60 copies of Epstein–Barr virus DNA per cell, was fractionated in neutral solution by several cycles of CsCl gradient centrifugation in fixed-angle rotors. Under the fractionation conditions used, intact Epstein–Barr virus DNA from virus particles can be separated from the less-dense cellular DNA. In contrast, a large proportion of the intrinsic Epstein–Barr virus DNA component of Raji cells remains associated with cellular DNA, as determined by nucleic acid hybridization. This interaction, which is resistant to Pronase and phenol treatment, is not the result of aggregation. When the molecular weight of Raji DNA is reduced by hydrodynamic shear, the amount of virus DNA associated with cell DNA decreases. However, some virus DNA still remains bound to fragments of cellular DNA after shearing. The association is completely destroyed in alkaline solution. Molecular weight analysis of Raji DNA after denaturation showed that the alkali-induced release of Epstein–Barr virus DNA was specific and not the result of random single-strand breaks. These data indicate that Epstein–Barr virus DNA is linearly integrated into Raji cell DNA by alkali-labile bonds.

Established human lymphoblastoid cell lines of the B-cell type contain several copies of Epstein–Barr virus (EBV) genome per cell (1–4), and it appears that only such virus-transformed cells have the capacity to grow in long-term culture (for review, see refs. 5 and 6). The amount of EBV DNA present has been quantitated by nucleic-acid hybridization techniques, with equivalent results being obtained both by filter hybridization with EBV complementary RNA and by DNA–DNA reassociation kinetics (4). For any given cell line, the number of EBV genomes per cell is a constant characteristic of that particular line, but different cell lines contain different amounts of virus DNA (3). Thus, cell line Raji, which was established from a Burkitt’s lymphoma biopsy (7) and is free from detectable amounts of EBV particles or antigens associated with virus production (8), contains 50–60 EBV genome equivalents per cell (refs. 2–4; this work).

The physical state of the resident EBV genomes in lymphoblastoid cells has remained unclear. By alkaline sucrose gradient centrifugation of very-high-molecular-weight cellular DNA, followed by localization of viral sequences by nucleic-acid hybridization, it was demonstrated that simian virus 40 (SV40) DNA is covalently integrated into host DNA in SV40-transformed cells (9). In applying this technique to Raji cells, however, Nonoyama and Pagano (10) found no detectable EBV DNA associated with the cellular DNA. Instead, the intracellular EBV DNA had the same sedimentation properties as alkali-denatured DNA isolated from virus particles. It was therefore proposed that EBV DNA molecules may exist as plasmids in virus-transformed cells, with an unusual type of integration remaining as an alternative explanation of the data. Here we describe experiments performed in neutral solution that indicate that there is a linear association between EBV DNA and cellular DNA in transformed human lymphocytes.

MATERIALS AND METHODS

Reagents. [3H]Thymidine (14 Ci/mm) and [3H]thymidine (54 Ci/mol) were purchased from NEN Chemicals. Pancreatic DNase (RNase free) and pancreatic RNase (DNase free) were from Worthington. Pronase (Calbiochem) was preincubated as a 1% solution in 50 mM Tris·HCl (pH 7.5) for 2 hr at 37°C before use. Escherichia coli [3P]DNA (106 cpm/μg), phage T7 [3P]DNA (106 cpm/μg), [3H]thymidine-labeled E. coli DNA (104 cpm/μg), and phage T7 [3H]DNA (8 × 104 cpm/μg) were prepared by standard methods. Standard saline citrate is 0.15 M NaCl–0.015 M trisodium citrate.

Virus DNA. [3H]Thymidine-labeled EBV was recovered from the culture medium of the virus-producing P3HR-1 cell line and purified as described (11). In some experiments the virus was further fractionated by sucrose gradient centrifugation. DNase-treated EBV concentrates in phosphate-buffered saline (pH 7.4) were lysed by addition of 0.05 volume of 1 M Tris·HCl–0.2 M EDTA (pH 8.5) and 0.02 volume of 25% Sarcosyl (Geigy), followed by heating at 55°C for 5 min. The solution was then incubated at 37°C with 1 mg/ml of Pronase for 2 hr and adjusted to a density of 1.710 g/cm³ by addition of solid CsCl. Viral DNA was quantitatively freed from any contaminating cellular DNA by centrifugation to equilibrium in a fixed-angle rotor.

Cellular DNA. Raji cells were carried as suspension cultures in minimal essential medium plus 15% fetal-bovine serum. Cultures were labeled with [3H]thymidine (0.02 μCi/ml) on the second day after refeeding, at a time when most cells were destined to go through at least one additional S phase. Cells from stationary cultures were collected by low-speed centrifugation and washed twice with phosphate-buffered saline. The cells were suspended at a concentration of 107 cells per ml in phosphate-buffered saline and lysed by addition of 0.5 volume of 3% Sarcosyl in 75 mM Tris·HCl–25 mM EDTA

Abbreviations: EBV, Epstein–Barr virus; SV40, simian virus 40; standard saline citrate, 0.15 M NaCl–0.015 M trisodium citrate.
and to fractionate sufficient quantities of such high-molecular-weight DNA to permit localization of the virus DNA sequences by nucleic-acid hybridization.

CsCl gradient centrifugation of DNA in fixed-angle head rotors gives better resolution and loading capacity than centrifugation in conventional swinging-bucket rotors (12). However, when DNA of molecular weight 1 to 2 × 10^9 is fractionated, the capacity of even such gradients is markedly reduced. In order to avoid aggregation and extreme collection artifacts, it was found in model experiments that the initial DNA concentration should be less than 15 μg/ml at 20°C. Fig. 1a shows the fractionation under such experimental conditions of a mixture of EBV [3H]DNA isolated from virus particles and a 1000-fold excess of unlabeled DNA isolated from Raji cells. The amount of virus DNA added to the Raji cells was about the same as that already present as an intrinsic component in these virus-transformed cells. The EBV [3H]DNA did not associate with cellular DNA to a detectable extent, but appeared as a symmetrical peak at the expected buoyant density of 1.718 g/cm³. The intactness of the EBV [3H]DNA at the end of this experiment was demonstrated by molecular-weight analysis on material from an identical duplicate CsCl gradient. It was observed by sedimentation at T7 [3P]DNA in a neutral sucrose gradient that 70% of the EBV [3H]DNA was of molecular weight 100 × 10^6, the size of intact molecules (19), with the remainder accounted for as half molecules. Raji cell DNA was analyzed separately, and was found to have a molecular weight after CsCl gradient centrifugation of 160 × 10^6.

Density Distribution of the Intrinsic EBV DNA in Raji Cells. Raji cell DNA (without added EBV DNA) was fractionated as in Fig. 1a, and the resident virus DNA sequences were localized by hybridization with EBV complementary RNA (Fig. 1b). Most of the virus DNA again appeared as a peak at a higher density than that of cellular DNA, but the profile of this intrinsic EBV DNA component differs in two respects from that of free virus DNA: (i) there is a small density shift, as the peak of the intrinsic virus DNA appears at a density of 1.716 g/cm³, and (ii) there is a pronounced tail of EBV DNA into the cellular DNA peak. From theoretical calculations (Eq. 2 in ref. 20) such a density profile would result if a large proportion (>50%) of the virus DNA sequences were linearly associated with fragments of cellular DNA.

In both gradients in Fig. 1 the peak of cellular DNA is asymmetrical. This finding is probably due to convection that occurs on collection of this very viscous DNA band. Part of this difficulty was attributed to the acuteness of the angle (23°) of the tube with respect to the axis of rotation in the type 60 Ti rotor, and in subsequent experiments the type 42 rotor, in which the tubes form a 30° angle, was used.

Isolation of Hybrid Viral–Cellular DNA. In order to further characterize the association between the EBV DNA component and the cellular DNA in Raji cells, DNA from 3 × 10^9 cells was fractionated on 14 large CsCl gradients. To facilitate the location of the DNA, the cells were labeled at a low specific activity with [3H]thymidine. A trace of [3P]-labeled, sheared E. coli DNA was included in some gradients as a density reference. Material from corresponding density regions of the 14 preparative gradients were combined into fractions I, II, and III, as indicated for a typical gradient in Fig. 2.

RESULTS

Separation of EBV DNA from Cell DNA. EBV DNA and human cellular DNA have different base compositions. Consequently, EBV DNA (ρ = 1.718 g/cm³, ref. 17) can be separated from host DNA (ρ = 1.700 g/cm³, ref. 18) by density gradient sedimentation in CsCl. It is thus possible to test if intracellular virus DNA is associated with cellular DNA sequences by determining the density distribution of the EBV DNA component present in transformed cells. For this approach, it is necessary to isolate DNA of similar or greater length than the EBV genome (molecular weight 10^9),

Fig. 1. Separation of EBV DNA sequences from Raji cell DNA by gradient centrifugation in neutral CsCl. (a) Profile of an artificial mixture of EBV DNA and Raji DNA. 0.3 µg of [3H]thymidine-labeled EBV DNA was added to 40 × 10⁶ living Raji cells in phosphate-buffered saline, together with Sarcosyl and Tris-EDTA (pH 9). After RNase and Pronase treatment of the lysate, the material was mixed with solid CsCl and 0.05 M Tris-HCl (pH 7.5) to give 35 ml of a solution of density 1.710 g/cm³. This DNA solution was divided equally between two tubes and centrifuged to equilibrium in the Spinco type 60 Ti rotor. From each tube, 35 fractions were collected. For one gradient, radioactivity and total DNA content (by the diphenylamine reaction) were estimated on separate aliquots of each fraction. Material from the viral and cellular DNA fractions of the duplicate gradient were used for molecular-weight analysis. □, µg of DNA; ▲—▲, cpm EBV [3H]DNA.
(b) Profile of resident EBV DNA in Raji cells as determined by DNA–complementary RNA hybridization. Another sample of 40 × 10⁶ Raji cells were lysed and fractionated as in (a), except that no exogenous EBV DNA was added. DNA from individual fractions was fixed on membrane filters and hybridized with EBV complementary [32P]RNA. •—•, µg of DNA; ▲—▲, complementary RNA hybridized.

Fractions I, II, and III were individually rebanded in CsCl a second time, and such material was used for further experiments. Thus, fraction II contained DNA of a density intermediate between that of EBV DNA and cellular DNA, while fraction III contained DNA with the density of cellular DNA. In agreement with the results in Fig. 1b, hybridization analysis of the material from the different density regions revealed that 85% of the intrinsic virus DNA had a density greater than 1.713 g/cm³. When individual fractions from the rebanded fraction I were analyzed, the EBV DNA component was again found at a density of 1.716 g/cm³, as in Fig. 1b.

Fig. 2. Fractionation of Raji cell DNA into various density components. A typical preparative CsCl gradient is shown. Centrifugations were done in a Spinco type 42 fixed-angle rotor, with 17 ml of DNA solution per tube. To some gradients, 10 ng of E. coli [32P]DNA was added as a reference density marker (ρ = 1.710 g/cm³). Material from this and the other 13 gradients was combined into three large fractions as indicated in the figure.

The persistent presence of EBV DNA sequences in DNA of a lighter density than that of free virus DNA indicates that EBV DNA and cell DNA are bound to each other in the form of hybrid DNA molecules. In attempts to disrupt this association, material from fraction III (ρ = 1.697–1.705 g/cm³) was centrifuged in CsCl a third time, both at a lower DNA concentration (3 µg/ml), and after dialysis and phenol extraction of the DNA. The association between EBV DNA and cell DNA remained stable after such treatments, and no EBV DNA (<15% of total) was detected by hybridization at the density of free virus DNA in these refractionation experiments. It therefore appears very unlikely that the association observed could be due to concentration-dependent DNA aggregation, or to protein remaining bound to the virus DNA.

Shear Sensitivity of Hybrid Viral–Cellular DNA. Fraction II and fraction III were divided in two parts, and one part of each solution was sheared by several passages through a syringe needle. The molecular weights of the DNA solutions before and after sheare were determined for fraction III; they were 110 × 10⁶ for unsheared DNA and 8.3 × 10⁶ for sheared DNA. The different DNA solutions were again fractionated in CsCl gradients, and the location of the virus DNA sequences was determined by hybridization. The profiles of total DNA and EBV DNA in each case are shown in Fig. 3.

The material in fraction II was enriched for sequences of cellular DNA of unusually high density. The EBV component formed a broad band at a density of 1.713 g/cm³ in the unsheared DNA, but after shearing the virus DNA appeared at a density of 1.718 g/cm³. Thus, in the sheared DNA solution most of the virus DNA component was released, and banded at the density of free virus DNA.

In fraction III the ratio of EBV DNA to cellular DNA was 0.01 of that in fraction II. The small amount of virus DNA
Fig. 3. Third sequential fractionation in CsCl of low-density EBV DNA in Raji cells. Effect of size reduction of DNA by hydrodynamic shear. In each case, 4.5 ml of DNA solution was centrifuged to equilibrium in a Spinco type 40 rotor, and each fraction was analyzed for total DNA and for EBV DNA. •, $A_{260}$; ▲, EBV complementary DNA hybridized. (a) Fraction II, high-molecular-weight DNA; (b) same material, low-molecular-weight DNA; (c) fraction III, high-molecular-weight DNA; (d) same material, low-molecular-weight DNA.

present banded at a density of 1.703 g/cm$^3$ in the unsheared solution (Fig. 3e). This low density for the virus DNA component indicates that the hybrid molecules in this material contained $>$90% cellular DNA. After shear, the EBV DNA component banded at a higher density, 1.711 g/cm$^3$, and very little material as heavy as free virus DNA was generated (Fig. 3d). It thus appears that most of the EBV DNA remained bound to cellular DNA, which is consistent with only short segments of virus DNA being associated with the unsheared cellular DNA. These data on fraction III are most readily interpreted as reflecting a linear integration of EBV DNA into cellular DNA, with the creation by shearing of short hybrid DNA molecules enriched in EBV DNA sequences.

**Alkali Lability of the Association.** An aliquot of unsheared DNA of fraction III was adjusted to pH 12.3 with NaOH and centrifuged to equilibrium in an alkaline CsCl gradient, in the presence of a trace of E. coli [$^3$H]DNA as a density reference. The DNA appeared as a symmetrical peak at the density expected for cellular DNA (Fig. 4). By hybridization of each fraction it was demonstrated that the EBV DNA component was separated from the cell DNA in this case, and was present at the approximate density of free virus DNA. No detectable virus DNA ($<$10%) remained associated with the cellular DNA (Fig. 4). Thus, the association between these two DNAs present in the neutral solution is apparently completely disrupted in alkaline solution.

A trivial explanation of these data would be that the high-molecular-weight DNA in fraction III contained numerous single-strand breaks. This was not the case, however, as shown by the size distribution of the material in alkaline solution (Fig. 5). The Raji [$^4$C]DNA in fraction III, which had a molecular weight of $110 \times 10^6$ in neutral solution, varied in size in alkaline solution from $10 \times 10^6$ to $50 \times 10^6$. Thus, some intact single strands were present, and most strands only con-

Fig. 4. Alkaline CsCl gradient centrifugation of DNA from fraction III. A 4.5-ml aliquot of this DNA fraction was adjusted to a density of 1.77 g/cm$^3$ with CsCl and to pH 12.3 with 1 M NaOH-0.03 M EDTA and was centrifuged to equilibrium (13). •, total DNA ([$^4$C]-labeled); ▲, EBV DNA sequences. Arrow shows the position of a density marker, E. coli [$^3$H]DNA. A small aliquot of each fraction was analyzed for $^3$H and $^4$C radioactivity, and the remaining material was hybridized with EBV complementary [$^3$H]RNA.
reflect aggregation with cellular DNA. 40% the resistant DNA, in fact, contained 1–2 interruptions. In Fig. 3d, double-stranded DNA fragments of molecular weight $8.3 \times 10^6$ still existed as hybrid molecules comprised of, on the average, 60% EBV DNA and 40% cellular DNA. It would therefore be necessary to postulate the presence of more than 10 single-strand breaks per chain in the DNA in fraction III in order to explain the alkali sensitivity of the association with EBV DNA on the basis of random single-strand breakage, which is clearly inconsistent with the data in Fig. 5.

**DISCUSSION**

The present results show that the intrinsic EBV DNA in Raji cells is tightly associated with the host-cell DNA. After purification of the resident EBV DNA component that had a density markedly different from that of free virus DNA by high-resolution CsCl gradient centrifugation, it was possible to demonstrate that shearing of this DNA to smaller fragments induced density shifts in the EBV DNA component. The density changes observed were in good agreement with those expected for EBV DNA being linearly integrated into the host-cell DNA, and the data thus offer strong support for this model.

The association between cellular DNA and EBV DNA was resistant to dilution, prolonged storage in solution, and treatment with phenol, Pronase, detergents, and EDTA. This makes it seem quite unlikely that the association would only reflect aggregation of the DNA in vitro. Moreover, extrinsic EBV DNA added to Raji cells at the moment of cell lysis did not bind to the cell DNA in a similar fashion. It has been reported that in Raji metaphase cells, all the EBV DNA is found in the chromosomes (10), and in situ hybridization experiments suggest that virus DNA is associated with several different chromosomes (21). Here, the density distribution of the intrinsic EBV DNA component in Raji cells shows that a large proportion of the virus DNA molecules is directly associated with cellular DNA sequences. Thus, the 50–60 EBV genomes present in Raji cells can not be integrated at one or a very few sites in the host-cell genome, but must be bound at many different chromosomal loci.

In agreement with the previous studies of Nonoyama and Pagano (10), no alkali-resistant association between EBV DNA and Raji cell DNA was observed. It would therefore appear that the mechanism of integration of EBV DNA is different from that found for SV40 (9) or adenoviruses (22), in that it does not involve DNA–DNA covalent linkage. Possibly, "linkers" of a different structure are present, e.g., short chains of RNA, or alternatively the cellular and viral DNA are only united by hydrogen bonds (20). It is of interest in this regard that about 10 covalently inserted ribonucleotide residues have recently been found in circular DNA molecules from human mitochondria (23), and that a small number of single-strand interruptions that are alkali labile are present in Herpes simplex DNA (24).

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