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Integrated Viral DNA Sequences in Epstein-Barr Virus-Converted Human Lymphoma Lines

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Most human lymphoid cell lines contain multiple copies of circular, nonintegrated Epstein-Barr virus (EBV) DNA molecules as well as viral DNA sequences with properties of integrated DNA. The physical state of the EBV DNA in a human lymphoma line that only contains one virus genome equivalent per cell has now been studied by three different methods, neutral CsCl density gradient centrifugation, actinomycin D-CsCl gradient centrifugation, and Hirt fractionation. This cell line, AW-Ramos, has been obtained by EBV infection in vitro of the apparently EBV-negative Ramos lymphoma line. The results indicate that the EBV DNA in AW-Ramos is present exclusively in a linearly integrated form. Similar data were obtained with two other EBV-converted sublines of Ramos cells.

Human lymphoid cells cannot be grown in long-term tissue culture, as a rule, unless the cells have been transformed by Epstein-Barr virus (EBV; for reviews, see 27, 38). Lymphoid cell lines derived from blood of healthy donors always are of the B (bone marrow-derived)-cell type, invariably contain multiple genome copies of EBV DNA, and express an EBV-associated nuclear antigen, EBNA. Similar EBV-transformed B-cell lines can be obtained at a high frequency from blood of infectious mononucleosis patients or from Burkitt lymphoma biopsies. Although the establishment of EBV-negative human lymphoid cell lines from nonmalignant sources has never been reported, biopsies from a few undifferentiated, highly malignant human B-cell lymphomas have yielded cell lines that appear to be free from EBV, as judged by EBNA negativity (22) and by absence of detectable amounts of EBV DNA (3). Two such EBV-negative lymphoma lines, Ramos and BJAB, are of particular interest because they can be infected with EBV in vitro and converted to EBNA-positive sublines (7, 13, 21). These EBV-converted lines contain small but detectable amounts of viral DNA, and one line of this type, AW-Ramos, only has about one EBV genome equivalent per cell (3). AW-Ramos cells are stably converted by EBV, and EBNA-negative cells do not appear in cultures at a detectable frequency.

The latent EBV DNA in typical human lymphoid cell lines is mainly present as circular DNA molecules of viral genome length (17, 23), but integrated viral DNA sequences also seem to be present (1, 2, 17). In this work, we have extended the studies on the physical state of intracellular EBV DNA to EBV-converted sublines of Ramos that carry small amounts of viral DNA. We have used three different methods suitable for the analysis of integration of high-molecular-weight viral DNA and show that such lines contain EBV DNA sequences with the expected properties of integrated DNA, whereas circular, nonintegrated viral DNA molecules appear to be absent.

MATERIALS AND METHODS

Cell lines. The human lymphoma-derived B-cell lines Raji (10), Ramos, AW-Ramos clone 2, EHRA-Ramos (21), Ramos/B95-8 (13), and U-698 M (22) were obtained from G. Klein, Department of Tumor Biology, Karolinska Institute, Stockholm. The cells were grown at 37°C in suspension culture in RPMI 1640 medium (Grand Island Biological Co.) supplemented with 15% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Actively growing cell cultures, containing 5×10^5 to 1×10^6 cells/ml and more than 85% living cells according to the trypan blue exclusion test, were used in all experiments. The cells were harvested by low-speed centrifugation and washed twice in 0.01 M sodium phosphate, pH 7.4, immediately before use.

DNA preparations and hybridization procedures. High-molecular-weight cellular DNA for gradient centrifugation experiments was obtained by lysis of a cell suspension containing 10^7 cells/ml in 0.01 M sodium phosphate (pH 7.4) by addition of 0.5 volume of 0.075 M Tris-hydrochloride (pH 8.5), 0.025 M EDTA, and 1.5% sodium dodecyl sulfate. After 20 min at 20°C, 0.2 volume of 0.5% Pronase (Calbiochem;

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self-digested in 0.05 M Tris-hydrochloride [pH 7.5] at 46°C for 45 min before use) was added, and the lysate was incubated at 37°C for 8 h (25). The viscous solution was then brought to 20°C, diluted with an equal volume of 0.1 M NaCl–0.075 M Tris-hydrochloride (pH 8.0), and extracted with 1 volume of freshly redistilled phenol equilibrated with the same buffer. The phenol extraction was performed by slowly pouring the mixture back and forth between two glass beakers. The mixture was then chilled to 8°C, and the two phases were separated by centrifugation. The aqueous phase was extracted once more with phenol in the same fashion and then dialyzed against 0.1 M NaCl–0.01 M Tris-hydrochloride (pH 8.0)–0.001 M EDTA for 48 h at 2°C, followed by dialysis for 4 h against the same buffer without NaCl.

Nonradioactive and ^{3}H-labeled (10^5 cpm/μg) EBV DNA preparations from virus particles released by the P3HR-1 cell line were gifts from A. Adams. The viral DNA preparations had more than 85% of the DNA in intact, 59S form. *Klebsiella pneumoniae* [^{3}H]DNA (10^5 cpm/μg, ρ = 1.717 g/cm^3, molecular weight = 3×10^7) was prepared by standard methods.

The preparation of ^{32}P-labeled EBV complementary RNA (cRNA) and the conditions for DNA-cRNA hybridization were as described (23).

CsCl density gradient centrifugation. For CsCl gradient centrifugation, DNA solutions were diluted to low concentrations, 2 to 6 μg/ml, and supplemented with solid CsCl (Merck Suprapur) to a final density of 1.710 to 1.714 g/cm^3 and a trace amount of *K. pneumoniae* [^{3}H]DNA. The DNA solutions were centrifuged in 18.5-ml samples in a Spinco 60Ti rotor at 33,000 rpm and 21°C for 65 h. Fractions (0.4 ml) were collected through a large hole in the bottom of the tube with the aid of a closed-system collection device and analyzed for refractive index, radioactivity, absorbancy at 260 nm, and hybridizability with EBV [^{32}P]cRNA as described (23). Further analyses of CsCl gradient fractions by neutral glycerol gradient centrifugation were also performed as described (23).

Actinomycin D-CsCl gradient centrifugation. The experiments were performed essentially according to Birnstiel et al. (5). DNA solutions were extensively dialyzed against 0.05 M sodium tetraborate (pH 9.0) at 3°C and diluted to a DNA concentration of 2 μg/ml. Fourteen milliliters of each DNA solution was supplemented with a trace amount of *K. pneumoniae* [^{3}H]DNA, 0.2 ml of a 0.2% actinomycin D (Sigma) solution, and solid CsCl to a refractive index of 1.3885 at 3°C. The solutions were then overlaid with paraffin oil and centrifuged in a Spinco 60Ti rotor at 33,000 rpm and 3°C for 96 h. Collection and analysis of fractions were as for CsCl gradients without actinomycin D, except that each fraction was diluted with an equal volume of 0.05 M sodium tetraborate (pH 9.0) before denaturation for hybridization analysis.

Hirt fractionation procedure. The fractionation was performed after Pronase treatment (16) and at a relatively low cell concentration (4). Cells (2×10^7 living cells) were suspended in 8 ml of 0.13 M NaCl–0.01 M sodium phosphate (pH 7.4). An equal volume of 1.2% sodium dodecyl sulfate–0.01 M Tris-hydrochloride (pH 8.0)–0.01 M EDTA was added at 20°C, and the solutions were gently mixed. After 60 min, 4 ml of preincubated 0.5% Pronase was slowly added, followed by incubation at 37°C for 4 h. The solution was then chilled to 0°C and gently mixed with 6.7 ml of 4 M NaCl–0.4 M Tris-hydrochloride (pH 7.6) (at 0°C). After 12 h at 0°C, the mixture was centrifuged at 24,000 $\times$ *g* for 45 min. The supernatant was recovered and dialyzed against 0.1 M Tris-hydrochloride (pH 8.0)–0.001 M EDTA, and the precipitate was dissolved in the same buffer at 20°C. The solutions were separately extracted with 1 volume of phenol at 20°C and freed from phenol by centrifugation and dialysis of the aqueous phase against 1 M NaCl–0.05 M Tris-hydrochloride (pH 8.0)–0.001 M EDTA for 48 h, followed by dialysis against the same buffer without NaCl. Several samples (10 to 12 μg each) of the DNA solutions were then denatured by incubation with an equal volume of 0.5 M NaOH at 80°C for 10 min, and this procedure also served to cleave large, covalently closed circular DNA molecules and to degrade contaminating RNA. The DNA was subsequently fixed to membrane filters and analyzed by hybridization with EBV [^{32}P]cRNA. The DNA-containing filters were recovered after the radioactivity measurements, and the DNA on each filter was acid hydrolyzed and quantitated by the diphenylamine reaction as described (23). The latter values were used to correct the hybridization data to 10.0 μg of DNA per filter.

RESULTS

Distribution of integrated viral DNA sequences after CsCl density gradient centrifugation. DNA molecules of different base composition can be fractionated by CsCl density gradient centrifugation, and this method has often been used to separate free viral DNA and host DNA and to study the integration of viral DNA (for reviews, see 9, 24). In its simplest form, the method can be used to measure the integration of a small viral genome, or a fragment of a virus DNA molecule, into a large piece of host DNA. In this case, the integrated viral DNA sequences will exhibit a density very close to that of the cellular DNA, and it has been shown in this fashion that fragments of adenovirus 12 DNA are integrated into cellular DNA after abortive infection of baby hamster kidney cells grown in the presence of bromodeoxyuridine (8), and that the DNA provirus of spleen necrosis virus is integrated into the chicken cell genome (14). When the virus genome is large, as in the case of a herpesvirus DNA molecule, the analysis becomes slightly more complicated, because standard methods for the preparation of high-molecular-weight DNA from mammalian cells using Pronase and phenol treatments (33) yield shear-produced large fragments of the host chromosome that are of a molecular weight close to 10^8, i.e., similar in size to the viral genome under study. In this case, integrated viral DNA sequences should be found at densities intermediate between those

of free viral DNA and host DNA after density gradient centrifugation if entire virus genomes are integrated, because random fragmentation of host chromosomes containing integrated virus genomes will result in the formation of "joint molecules" comprised of varying proportions of viral and cellular DNA sequences. With the assumption that the joints between viral and cellular DNA sequences are not anomalously shear sensitive, a typical joint molecule might then be comprised of approximately equal parts of viral and cellular DNA.

There is no method available to equally measure all such joint molecules. Instead, viral DNA sequences in joint molecules can be determined by nucleic acid hybridization, using a radioactive virus nucleic acid probe. This means that a joint molecule comprised of a long sequence of viral DNA and a short sequence of cellular DNA will provide more sequences complementary to the radioactive probe than a joint molecule comprised of a short sequence of viral DNA and a long sequence of cellular DNA. Consequently, even if free virus DNA molecules, cellular DNA "molecules," and viral-cellular DNA joint molecules are all of the same size, the profile obtained by hybridization with a viral nucleic acid probe over a CsCl gradient containing cellular DNA with integrated virus genomes will not yield a symmetric radioactivity peak at equal distance between viral and cellular DNA. Instead, a skewed peak localized closer to the position of free viral DNA than to cellular DNA would be expected.

When DNA molecules isolated from transformed cells and free viral genomes are of similar size, the determination of the expected distribution of integrated viral DNA after density gradient centrifugation becomes formally equivalent to the theoretical analysis of the density distribution of bacteriophage T4 recombinant DNA molecules performed by Tomizawa and Anraku (34). These authors infected cells simultaneously with bromouracil-containing, nonradioactive T4 particles of high density and a small amount of ^{32}P-labeled T4 phage of normal density, and registered joining of parental T4 DNA molecules by measuring the amounts of radioactive T4 DNA found at anomalous densities in CsCl gradients after extraction and fractionation of high-molecular-weight DNA from infected cells. In this connection, an equation was derived to show the expected distribution of radioactive residues in joint molecules (equation 2 in reference 34). Here, we have applied the Tomizawa-Anraku equation to estimate the expected density distribution in neutral CsCl gradients of EBV DNA integrated into human DNA. EBV DNA sequences can be separated from host cellular DNA in CsCl gradients because a large, natural density difference exists between the virus DNA (ρ = 1.718 g/cm^3) and mammalian DNA (ρ = 1.700 g/cm^3), whereas EBV DNA has little internal density heterogeneity (30). Figure 1 shows the experimentally determined density values for free EBV DNA from virus particles and for host DNA, as well as the expected theoretical distribution of integrated virus DNA sequences for the case of integration of entire EBV genomes as single entities. Whereas the free virus DNA is found as a narrow peak at a density of 1.718 g/cm^3, the integrated viral DNA would be detected as a broader, slightly skewed profile with a peak density of 1.713 g/cm^3 by hybridization with a viral nucleic acid probe. There are no detectable free viral DNA sequences at the density of cellular DNA, whereas a significant tail of integrated viral DNA sequences would be found at this density. The latter material would represent viral-cellular DNA joint molecules mainly consisting of cellular DNA. Several assumptions have been made in this analysis.

(i) All cellular DNA molecules have the same density. This is not entirely correct, but moderate density heterogeneity within the peak of cellular DNA would not markedly change the theoretical distribution profile of integrated viral DNA (34). It should be noted, however, that

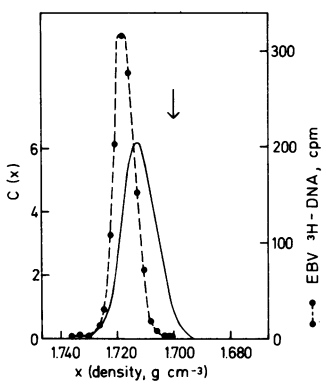

FIG. 1. *Estimate of the density distribution of integrated EBV DNA in a neutral CsCl gradient. The dashed line shows experimental data for EBV [^{3}H]DNA, isolated from virus particles, after centrifugation at 33,000 rpm in a Spinco 60Ti rotor for 65 h at 20°C in an 18.5-ml neutral CsCl gradient. The data can be approximated by a Gaussian distribution with a peak density of 1.718 g/cm^3 and σ = 0.004 g/cm^3. The arrow shows the peak position of human cell DNA (1.700 g/cm^3) as determined in a separate experiment. The solid line shows the theoretically expected distribution of EBV DNA in the form of viral-cellular joint molecules, c(x), as a function of density, calculated according to Tomizawa and Anraku (34). For further details, see text.*

the present estimate concerns viral DNA integrated into cellular DNA of typical base composition (ρ = 1.700 g/cm^3). It is obvious that different distributions would be expected for the unlikely cases of integration of EBV DNA into guanine-cytosine-rich DNA such as nucleolar DNA, or into satellite DNA of unusual base composition.

(ii) All DNA molecules are of the same length. This requirement is essentially followed in the present work, as the isolated cellular DNA was of similar or slightly larger size than intact EBV DNA (see below).

(iii) Fragmentation of cellular chromosomes containing integrated viral DNA occurs by random shear-induced breakage, so that ratios of viral to cellular DNA in joint molecules range from zero to infinity, and all values occur with equal probability.

(iv) Viral DNA is integrated in the form of entire but single genomes. Clearly, if the virus DNA is integrated as many small fragments of viral DNA at multiple positions, the integrated virus DNA sequences should be found at the density position of cellular DNA. On the other hand, if several EBV genomes are tandemly integrated, they would be difficult to distinguish from nonintegrated virus DNA by CsCl density gradient centrifugation.

(v) Differences in DNA methylation between EBV DNA from virus particles and intracellular EBV DNA, if they exist, would not be of such a magnitude as to cause marked differences in buoyant density (20). In support of this assumption, it is noted that intracellular nonintegrated EBV DNA molecules, isolated mainly as nicked circular DNA molecules, have densities very close to that of EBV DNA from virus particles (17).

(vi) The radioactive viral nucleic acid probe used to detect viral DNA sequences by hybridization experiments is equally representative of all sequences in the EBV genome. In the present work we have used ^{32}P-labeled EBV cRNA, made with *Escherichia coli* RNA polymerase (containing sigma factor), and such cRNA preparations have been shown to represent most or all of the EBV genome (3). However, it is possible that abundance differences between different sequences occur in the cRNA preparations.

As the above assumptions may not be strictly fulfilled in the present case, the theoretical distribution shown in Fig. 1 should only be regarded as an approximation. Nevertheless, it seems likely that this estimate is a considerably better representation of the expected location of long integrated sequences of EBV DNA than the simple assumption that all integrated herpesvirus DNA sequences might be found at the density of cellular DNA.

Fractionation of lymphoma cell DNA by neutral CsCl density gradient centrifugation. Three different EBNA-positive sublines of the EBV-negative Ramos cell lines were used. Two of these lines, AW-Ramos and EHRA-Ramos, were obtained by infection of Ramos cells with the P3HR-1 strain of EBV (21), whereas the third line, Ramos/B95-8, was obtained by infection with the B95-8 strain of EBV (13). In agreement with previous results (3, 12), the AW-Ramos cells were found to contain approximately one EBV genome equivalent per cell, as determined by DNA-cRNA hybridization, whereas EHRA-Ramos had four and Ramos/B95-8 had two EBV genome equivalents per cell. High-molecular-weight DNA was prepared from these lines by lysis of the cells with sodium dodecyl sulfate and EDTA, followed by prolonged treatment with a high concentration of Pronase, and finally two gentle phenol extractions followed by dialysis. The DNA was subsequently mixed with a trace amount of a density marker, *K. pneumoniae* [^{3}H]DNA, fractionated by neutral CsCl density gradient centrifugation, and collected under conditions minimizing shearing forces (23). Such DNA had a sedimentation coefficient of 62S, corresponding to a molecular weight of 1.1 $\times$ 10^8 (reference 11), as determined by cosedimentation of [^{3}H]DNA from AW-Ramos, recovered from a CsCl gradient, with phage T4 [^{14}C]DNA in a neutral glycerol gradient.

As a control, DNA was isolated from the extensively studied Raji cell line by the same procedures, and EBV DNA sequences were localized by hybridization of individual CsCl gradient fractions. This Burkitt lymphoma-derived line contains 50 to 60 EBV genome equivalents per cell (28), and most of this virus DNA is present as circular, nonintegrated DNA, but virus DNA sequences with properties of integrated DNA are also present (1). The data are shown in Fig. 2. The peak of EBV DNA sequences from Raji cells is found at a density of 1.716 g/cm^3, i.e., at a density lower than that of free viral DNA (1.718 g/cm^3) but at a density higher than that expected for singly integrated viral genomes (1.713 g/cm^3). Furthermore, the peak is slightly skewed in shape and extends into the density region of cellular DNA. These observations are consistent with the presence of both nonintegrated and integrated EBV DNA sequences in these cells. The hybridization profile obtained is representative of the intrinsic density of the intracellular EBV DNA, because it has been shown (2) that the radioactive viral DNA

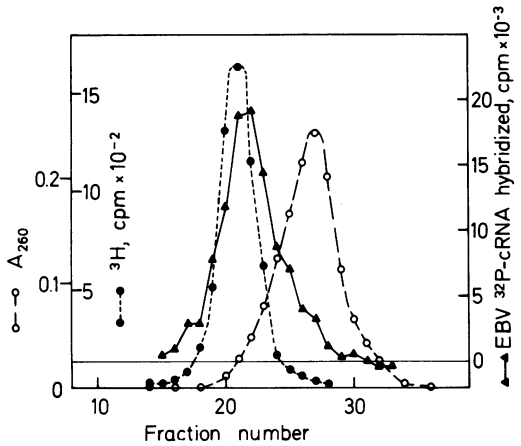

FIG. 2. *Fractionation of high-molecular-weight DNA from Raji cells by neutral CsCl density gradient centrifugation. DNA was extracted from the cells by lysis with sodium dodecyl sulfate and EDTA, followed by extensive treatment with Pronase. The DNA was then extracted twice with phenol, dialyzed, mixed with a trace amount of K. pneumoniae [^{3}H]DNA as density marker, diluted, and supplemented with solid CsCl to a final density of 1.710 to 1.714 g/cm^3. Each 18.5-ml gradient contained the DNA from 4 × 10^6 to 6 × 10^6 cells in 0.01 M Tris-hydrochloride–0.001 M EDTA (pH 8.0) and was centrifuged in a Spinco 60Ti rotor at 33,000 rpm and 20°C for 65 h. Fractions were slowly collected through a large hole in the bottom of the tube, and the cellular DNA and the density marker were localized by absorbancy at 260 nm (A_{260}) and radioactivity measurements. The DNA in the individual fractions was then alkali denatured and immobilized on membrane filters. Each filter was hybridized with 6 ng of EBV [^{32}P]cRNA (8.8 × 10^5 cpm after correction for ^{32}P decay), and the background of cRNA nonspecifically bound to filters containing heterologous DNA (800 cpm) was subtracted. The gradients were linear within the entire density range of interest (1.68 to 1.75 g/cm^3) as determined by refractive index measurements. Symbols: Cellular DNA of buoyant density 1.700 g/cm^3 (○); K. pneumoniae [^{3}H]DNA of buoyant density 1.717 g/cm^3 (●); EBV DNA sequences (▲).*

component in an artifical mixture of ^{3}H-labeled EBV DNA from virus particles and high-molecular-weight cellular DNA bands at the density of free viral DNA in neutral CsCl gradients, that the intracellular EBV DNA sequences from Raji cells found at an anomalously low density remain at the same density in rebanding experiments at low DNA concentrations, and that such low-density EBV DNA is also found after CsCl gradient centrifugation of Pronase-treated cell lysates not extracted with phenol.

Figure 3 shows a neutral CsCl density gradient fractionation experiment with high-molecular-weight DNA from AW-Ramos cells, which contain 50 times less EBV DNA than Raji cells. In this case, the EBV DNA sequences are found at a peak density of 1.713 g/cm^3, and the hybridization profile is similar to the theoretical estimate for an integrated EBV DNA molecule in Fig. 1. These data indicate that the viral DNA sequences in AW-Ramos are present in integrated form. The results do not seem compatible with a situation in which AW-Ramos cells contain one nonintegrated EBV DNA circle per cell of density of 1.718 g/cm^3. In agreement with this notion, no viral DNA with the sedimentation properties of covalently closed circular DNA of viral genome size was detected when the AW-Ramos DNA banding at a density of 1.710 to 1.718 g/cm^3 in CsCl gradients was further fractionated by neutral glycerol gradient centrifugation and analyzed by hybridization (data not shown). Moreover, the viral DNA in AW-Ramos cells is not present in the form of many small fragments integrated at several locations, as there are few EBV DNA sequences found at the density of cellular DNA. The data do not distinguish between the possibilities that AW-Ramos contains one integrated complete EBV genome or a couple of large fragments of viral DNA. AW-Ramos cells, in contrast to Raji cells, cannot be induced to express EBV antigens associated with an abortive lytic virus cycle by iododeoxyuridine treatment (21), and it is not known if all sequences of the EBV genome are present in AW-Ramos.

When DNA from AW-Ramos cells was reduced in size by shear treatment to a molecular weight of 6 × 10^6 before CsCl density gradient centrifugation, the EBV DNA sequences were instead found as a peak at 1.718 g/cm^3, i.e., the density of free viral DNA (3). This shows that

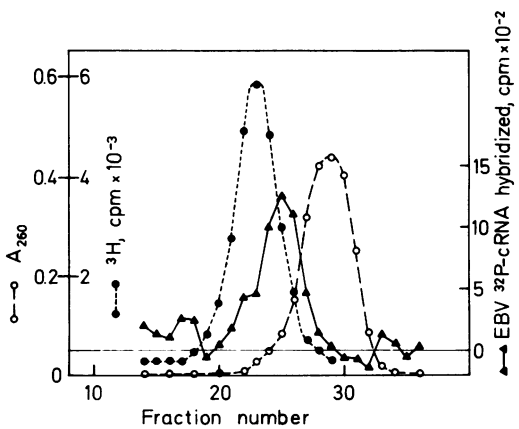

FIG. 3. *Fractionation of AW-Ramos DNA by neutral CsCl density gradient centrifugation. Experimental conditions and symbols as in Fig. 2.*

the low density observed for the intracellular EBV DNA after fractionation of high-molecular-weight DNA from AW-Ramos cells is not due to an unusual base composition of the viral DNA sequences present in the cells.

Results similar to those obtained with AW-Ramos were also observed on fractionation of high-molecular-weight DNA from the two other EBV-converted Ramos sublines, EHRA-Ramos and Ramos/B95-8, by CsCl density gradient centrifugation. In both cases, the EBV DNA sequences were detected as broad hybridization profiles at densities markedly lower (1.710 to 1.714 g/cm^3) than that of free EBV DNA (data not shown).

Actinomycin D-CsCl gradient centrifugation. In CsCl gradients containing actinomycin D, guanine-cytosine-rich DNA bands at a lower density than adenine-thymine-rich DNA (5, 18). Consequently, integrated EBV DNA would be expected to band at a higher density than free EBV DNA in such gradients, and the distribution of integrated viral DNA sequences would be approximated by a mirror image of Fig. 1.

When high-molecular-weight DNA from an EBV-negative human lymphoma line was mixed with small amounts of EBV DNA from virus particles and *K. pneumoniae* [^{3}H]DNA, the latter DNA serving as density marker, the results shown in Fig. 4 were obtained by actinomycin D-CsCl gradient centrifugation. Although *K. pneumoniae* DNA and EBV DNA have very similar base compositions, the EBV DNA banded at a slightly higher density than the bacterial DNA, presumably because of sequence effects. As expected, the human cell DNA was found at a markedly higher density than either of the two more guanine-cytosine-rich DNAs. The properties of high-molecular-weight AW-Ramos DNA, centrifuged together with *K. pneumoniae* DNA in the same fashion, are shown in Fig. 5. The separation between the bacterial DNA and the human cell DNA is similar to that observed in the control experiment. However, the intrinsic EBV DNA sequences of AW-Ramos are found at a significantly higher density than that of free viral DNA in this type of gradient. These data are in agreement with those shown in Fig. 3 and provide additional evidence for the presence of integrated EBV DNA sequences in AW-Ramos cells.

Hirt fractionation procedure. DNA from small tumor viruses can be efficiently separated from high-molecular-weight cellular DNA in cell lysates by coprecipitation of the latter DNA with sodium dodecyl sulfate in cold 1 M NaCl. The relative sizes of the DNA molecules are clearly important for the fractionation, and the

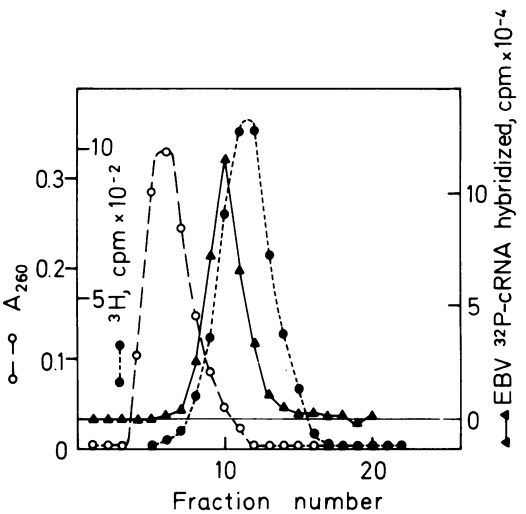

FIG. 4. *Actinomycin D-CsCl gradient centrifugation of human DNA from an EBV-negative lymphoma line, U-698M, mixed with trace amounts of EBV DNA from virus particles and K. pneumoniae [^{3}H]DNA. A 30-μg amount of the high-molecular-weight DNA was dialyzed against 0.05 M sodium tetraborate (pH 9.0), supplemented with actinomycin D and CsCl, and centrifuged in a final volume of 19 ml in a Spinco 60Ti rotor for 4 days at 33,000 rpm and 3°C. Fractions (0.4 ml) were collected from the bottom of the tube, followed by measurements on individual fractions of refractive index, absorbancy at 260 nm (A_{260}), ^{3}H radioactivity, and hybridizability with EBV [^{32}P]cRNA. Symbols as in Fig. 2.*

method is also applicable to Pronase-treated lysates (16). It has recently been found that molecules as large as intact herpesvirus DNA can be separated from cellular DNA in this fashion (4, 29). Here, we have used this procedure to obtain partial separation of nonintegrated EBV DNA from human cellular DNA.

In control experiments with phage T4 [^{14}C]DNA, which is similar in size to herpesvirus DNA, added in trace amounts to Pronase-treated lysates of human lymphoma cells, considerable trapping of the T4 DNA was observed. Using a relatively low cell concentration (10^6 cells/ml), which has been reported to facilitate the separation of herpesvirus DNA from cell DNA (4), we obtained 13 to 15% of the cellular DNA and 50 to 60% of the T4 DNA in the "Hirt supernatant." It is clear that the presence of viral DNA in the "Hirt precipitate" cannot be taken as evidence for integration when the virus genome has a molecular weight of 10^8, but it is also clear that the T4 DNA was enriched in the Hirt supernatant. Table 1 shows that when Raji cell DNA was fractionated in the same fashion, the Hirt supernatant was threefold enriched in EBV DNA sequences. These data indicate that

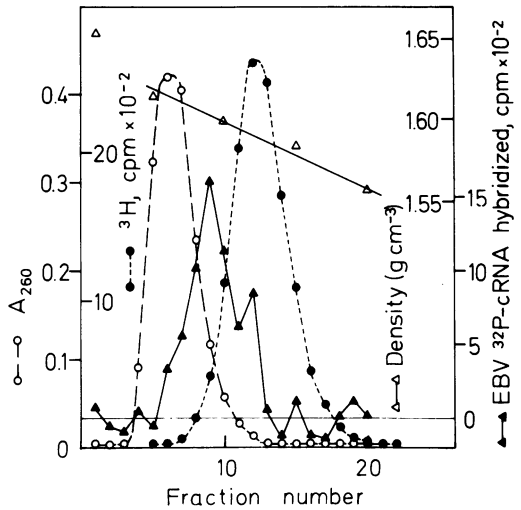

FIG. 5. *Analysis of intrinsic EBV DNA sequences in AW-Ramos cells by actinomycin D-CsCl gradient centrifugation. Experimental conditions as in Fig. 4 and symbols as in Fig. 2.*

some nonintegrated EBV DNA is present in Raji cells. There is much better evidence for this notion obtained by other techniques (23), and the EBV DNA present in the Hirt supernatant from Raji cells has been further purified and shown to consist largely of circular viral DNA molecules (data not shown). In contrast, there was no difference between the proportion of EBV DNA to cellular DNA between the Hirt supernatant and the corresponding precipitate from AW-Ramos cells (Table 1). These data are again consistent with the absence of nonintegrated EBV DNA molecules in AW-Ramos cells. Furthermore, the results indicate that the EBV DNA sequences in AW-Ramos cells are not present in the form of circular DNA molecules of viral genome size comprised of both cellular and viral DNA sequences.

DISCUSSION

Several different methods have been used to study the integration of tumor virus DNA sequences in virus-transformed host cells, and there are now many excellent techniques available to convincingly demonstrate the integration of small tumor virus genomes or fragments of virus DNA (6, 8, 15, 31, 36, 37). Unfortunately, most of these methods depend on the smaller size of the tumor virus genomes under study than of gently isolated cellular DNA, and they are not readily applicable to the characterization of intracellular forms of high-molecular-weight viral DNA. In that case, nonintegrated viral genomes may be found as a mixture of covalently closed circular DNA and nicked circular DNA, and one or the other of these forms is likely to have fractionation properties similar to those of the host DNA in different types of centrifugation experiments. In addition, methods that involve precipitation steps to separate viral and cellular DNA are marred by trapping artifacts that do not occur to a significant extent with small DNA molecules. When a large natural density difference between the viral DNA and host DNA exists, as in the present case, neutral CsCl density gradient centrifugation is a useful method for searching for large viral DNA molecules in integrated form. Alkaline CsCl gradients are less satisfactory for such studies on high-molecular-weight DNA because of the slow, salt-promoted hydrolysis of DNA that takes place in that solvent (19, 35). In the present work, the analysis of EBV-converted lymphoma lines that carry relatively small amounts of EBV DNA by neutral CsCl gradient centrifugation yielded results that are strongly indicative of the presence of integrated viral DNA sequences in the cells, whereas the data do not seem compatible with the occurrence of intracellular nonintegrated viral DNA. Similar results were obtained by centrifugation of AW-Ramos DNA in actinomycin D-CsCl gradients. Although the latter method does not seem to offer any gain in sensitivity over regular CsCl gradient centrifugation in the present case (Fig. 3 and 5), the data serve to confirm that the anomalous density properties of the intracellular viral DNA sequences are due to an association with DNA of relatively lower guanine-cytosine content.

The EBV-converted sublines of the Ramos lymphoma line have different growth characteristics and survive much better in crowded suspension cultures than the parent line (32), and they also express EBNA in contrast to the par-

TABLE 1. *Amounts of EBV DNA sequences in DNA from human lymphoma lines after Hirt fractionation*

Source of DNA	EBV [^{32}P]cRNA bound/10 μg of DNA (cpm)[a]	
	Hirt supernatant[b]	Hirt precipitate
Ramos	497	532
AW-Ramos	908	944
Raji	28,790	9,836

[a] Average values of triplicate determinations. Each 0.3-ml reaction mixture contained 2.5 ng (370,000 cpm) of EBV [^{32}P]cRNA.

[b] The Hirt supernatant fractions contained 14 ± 2% of the total cellular DNA, using a low initial cell concentration and Pronase treatment before fractionation.

ent line, so from this point of view lines such as AW-Ramos may be regarded as "EBV-transformed." On the other hand, the parental, apparently EBV-negative Ramos lymphoma cells have already been transformed in an unknown event, since they grow indefinitely in culture and show malignant potential on injection into nude mice (21). To avoid a nomenclature problem, the term "EBV conversion" has been used in the present work to describe the stable alteration of properties observed after EBV infection of an EBV-negative lymphoma line.

The integration of EBV DNA into the DNA of human lymphocytes does not appear to be sufficient by itself for transformation to malignancy, since small amounts of integrated EBV DNA sequences have been found in newly established human lymphoblastoid cell lines of diploid karyotype that do not cause tumors in adult nude mice (17, 26). It may be necessary to study the sites of viral DNA integration to evaluate the transforming potential of EBV, and such measurements will have to await the development of suitable technology for detailed characterization of integrated sequences of large viral DNA molecules.

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