Detection of Circular and Linear Herpesvirus DNA Molecules in Mammalian Cells by Gel Electrophoresis

THOMAS GARDELLA,1 PETER MEDVECZKY,2 TAKEI Sairenji,2 AND CAREL MULDER1,2*

Departments of Molecular Genetics and Microbiology1 and Pharmacology,2 University of Massachusetts Medical School, Worcester, Massachusetts 01605

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A simple gel technique is described for the detection of large, covalently closed, circular DNA molecules in eucaryotic cells. The procedure is based on the electrophoretic technique of Eckhardt (T. Eckhardt, Plasmid 1:584–588, 1978) for detecting bacterial plasmids and has been modified for the detection of circular and linear extrachromosomal herpesvirus genomes in mammalian cells. Gentle lysis of suspended cells in the well of an agarose gel followed by high-voltage electrophoresis allows separation of extrachromosomal DNA from the bulk of cellular DNA. Circular viral DNA from cells which carry the genomes of Epstein-Barr virus, Herpesvirus saimiri, and Herpesvirus atelos can be detected in these gels as sharp bands which comigrate with bacterial plasmid DNA of 208 kilobases. Epstein-Barr virus producer cell lines also show a sharp band of linear 160-kilobase DNA. The kinetics of the appearance of this linear band after induction of viral replication after temperature shift parallels the known kinetics of Epstein-Barr virus production in these cell lines. Hybridization of DNA after transfer to filters shows that the circular and linear DNA bands are virus specific and that as little as 0.25 Epstein-Barr virus genome per cell can be detected. The technique is simple, rapid, and sensitive and requires relatively low amounts of cells (0.5 × 10^6 to 2.5 × 10^6).

Covalently closed circles (CCC) of DNA such as bacterial chromosomes, plasmids, and some bacteriophages are commonly found in bacteria. In mammalian cells CCC are found both in the cytoplasm as mitochondrion DNA and in the nucleus of cells lytically infected with papovaviruses. Smaller CCC of unknown origin have been described (4, 25, 29) but have yet to be fully characterized. Epstein-Barr virus (EBV)-, Herpesvirus saimiri-, and Herpesvirus atelos-positive cell lines contain large numbers of extrachromosomal CCC of viral DNA, 150 to 200 kilobases (kb) in size (for reviews, see references 2 and 9). Viral DNA from particles of these three herpesviruses is a linear duplex ranging in size from 140 to 160 kb.

The search for CCC in mammalian cells has been hampered by the vast excess of chromosomal DNA. Therefore, CCC of DNA had to be partially purified before they could be detected, usually by electron microscopy. The enrichment has been done either by gentle DNA extraction followed by equilibrium centrifugation in CsCl-ethidium bromide (EtBr) gradients or by alkali extraction of nuclei at pH 12.4 followed by phenol extraction and phase separation of duplex circular and denatured DNA (10). The handling of DNA in these methods frequently causes single-strand breakage in a large proportion of high-molecular-weight CCC. Both methods have worked for cells from the three virus-associated tumors because of the large number of CCC in these cells, but relatively large amounts of cells and considerable efforts are required for such analyses.

For bacterial systems, the detection has been easier because even single copies of large CCC of plasmid DNA can constitute 5 to 20% of total cellular DNA. Eckhardt has developed a technique to detect these large molecules (6). He first converts the bacteria to protoplasts or spheroplasts in the well of an agarose gel and then subjects them to gentle lysis in situ by slowly electrophoresing sodium dodecyl sulfate (SDS) into the spheroplast suspension. The released DNA is then directly analyzed by high-voltage electrophoresis. The absence of any mechanical handling prevents most shear-induced breakage of the DNA: thus, the bulk of chromosomal DNA remains in large segments and is excluded from the gel. A small amount of cellular DNA which enters the gel and migrates as a broad linear DNA front probably arises from random fragmentation of a small fraction of the chromosomes. Plasmids ranging in size from 3.2 to 600 kb have been resolved as sharp bands between the linear DNA front and the origin (22). We have found that with minor modifications this method can be used successfully to detect CCC of DNA in mammalian cells, and we have used it to study the intracellular forms of herpesvirus DNA. The method is simple, requires only 0.5 × 10^6 to 2.5 × 10^6 cells, and results in the separation of circular forms of viral DNA from host chromosomal DNA. In addition, a separate band corresponding to linear viral DNA molecules can be detected at the bottom of the gel.

MATERIALS AND METHODS

Cells. Lymphoid cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Between 5 × 10^5 and 2 × 10^6 cells per ml were maintained in an atmosphere of 5% CO₂ in air at 37°C.

EBV DNA-carrying cell line Raji (gift from J. L. Sullivan) was derived from an African Burkitt lymphoma (20). Cell lines Jijoye and P3HR-1 are virus-producing cell lines. Jijoye was derived from an African Burkitt lymphoma (19), and P3HR-1 is a clonal line of Jijoye (14). Cell line QIMR-WIL was derived from a human myeloblastic leukemia patient (18).

Cell line 8402 (gift from J. L. Sullivan) is an EBV-negative T-cell line derived from a human leukemia patient (23).

Lymphoid cell lines 1670 and 70N2 (gift from B. Fleckenstein) were derived from tumors induced by H. saimiri 11 in tamarin marmoset monkeys (Saginus sp.).
Cell line 22CM37 was derived from cottontopped marmoset (Saguinus oedipus) lymphocytes, transformed in vitro by *H. atelis* 73 (8).

Cell line 5-78 (gift from M. D. Daniel) was derived from a lymphoma of ACCRB rabbits induced by *H. atelis* 810 (M. D. Daniel, R. D. Hunt, B. Fleckenstein, N. W. King, R. Tamulevich, D. Silva, and J. Ingells, manuscript in preparation).

The bacterium *Agrobacterium tumefaciens C58* (gift of W. Buikema) contains plasmids of 436 kb (M. Villarreal and M. Van Montagu, personal communication) and 208 kb (7) which serve as plasmid size markers. This strain was grown in Luria broth at 32°C.

**Buffers.** Electrophoresis buffer TBE contained 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA (pH 8.2).

Sample buffer A (for eucaryotic cells) contained 15% Ficoll, 2 Kunitz units of RNAase type I (Sigma Chemical Co.) per ml, and 0.01% bromophenol blue in buffer TBE.

Sample buffer B (for procaryotic cells) was sample buffer A to which lysozyme (Sigma) was added just before use to a final concentration of 7,500 U/ml.

Lysis buffer was buffer TBE containing 5% Ficoll, 1% SDS, 1 mg of pronase (added just before use) per ml, and 0.05% xylene cyanol green.

Phosphate-buffered saline (PBS) contained 2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, and 8 mM Na₂HPO₄.

Transfer buffer for transferring DNA from gels to APT paper was 20 mM sodium acetate adjusted with 20 mM acetic acid to pH 4.0, and for transferring DNA to cellulose nitrate, it was 1.5 M NaCl in 0.5 M Tris-hydrochloride (pH 7.0).

SSC was 0.15 M NaCl plus 0.015 M sodium citrate (pH 7.5).

**Sample preparation.** Eucaryotic cells (between 5 × 10⁸ and 2.5 × 10⁹) which were viable to more than 85% as judged by the trypan blue exclusion test were sedimented at 2,000 × g for 1 min. Cells were resuspended in 0.1 ml of sample buffer A either directly or after one washing in PBS.

For bacterial cells, ca. 6 × 10¹² cells from an exponentially growing culture were sedimented and resuspended in 0.1 ml of sample buffer B.

**Cell lysis and electrophoresis.** Sample loading and electrophoresis were carried out at 4°C. The cell suspension (0.075 ml) was pipetted into the well (10 by 10 by 2.5 mm) of a vertical 0.8% agarose gel (120 by 120 by 2.5 mm) prepared in buffer TBE. Lysis buffer (0.1 ml) was then carefully layered over the cell sample layers. Electrophoresis was started at 0.8 V/cm for 3 h and then increased to 7.5 V/cm for 14 h. The electrophoresis buffer was recirculated for the entire 17 h. Gels were stained with EtBr (1 µg/ml) (1) and photographed under UV light, using a Kodak 23A filter and Polaroid 775 film.

**Horizontal gels.** Horizontal gels were prepared in two steps. (i) Agarose (0.75%) in buffer TBE was poured onto a plastic plate (18 by 28 cm) surrounded by bars. A plastic bar (16 by 2 by 4 [height] cm) was placed 3 cm from the short end of the plate. Gels were usually 7- to 8-mm thick but were sometimes thicker to accommodate larger amounts of cells. (ii) After the agarose was solidified, the bar was removed, and a comb (14 by 0.7 cm) was placed in the resulting cutout against the agarose, i.e., 5 cm from the short end of the gel. The remaining area of the trough was filled with 0.8% agarose in TBE with 2% SDS to which pronase (final concentration, 1 mg/ml) had been added after the agarose had cooled to 50°C. After 30 min, the comb was removed, and the gel was transported to a 4°C area. Wells were loaded with suspensions of cells in 15% Ficoll, and electrophoresis (3 h at 0.8 V/cm) was started immediately to avoid SDS diffusion. After electrophoresis (24 h at 4.5 V/cm), the gel was washed with gentle rocking three times for 30 min in 1 liter of 10 mM sodium phosphate (pH 7.0). The gel was then stained with EtBr as described above.

**Hybridizations.** The DNA in the gel was partially depurinated (8 min in 250 ml of 0.25 M HCl), melted in 0.5 M NaOH in 1.5 M NaCl, neutralized, and transferred to aminophenylthioether (APT) paper or to nitrocellulose filters (26) in transfer buffer under partial vacuum (P. Medveczky and C. Mulder, manuscript in preparation). The papers were hybridized in 10 ml of hybridization buffer containing 35% formamide, 5 × SSC, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll, 15 mM sodium phosphate (pH 6.5), 10% dextran sulfate, 0.01% calf thymus DNA, and 10 ng of DNA probes labeled by nick translation (21) with [³²P]dCTP (NEG 013H; New England Nuclear Corp.) to a specific activity of 2 × 10⁹ to 5 × 10¹⁰ cpm/µg.

The DNA probe was either purified *H. atelis* DNA (gift of B. Fleckenstein) or bacterial plasmids containing segments of viral DNA. The plasmids used were pBam A (EBV BamHI-A in pBR322), pDK-52 (EBV BamHI-W in pBR322), and pSP47 (pB322 containing *H. saimiri* 11 H-DNA linked to the left-most 14 kb of its L-DNA). Plasmids were kindly supplied by E. Kieff (pDK-52), J. Skare (pBam A), and E. Szomolanyi (pSP47). Hybridizations were performed for 16 h at 42°C. Each filter was then washed four times in 250 ml of 0.1 × SSC–0.1% SDS for 1 h at 42°C and exposed to Kodak XAR-2 film for 12 to 24 h at −70°C, using a Dupont Cronex Lightning-Plus intensifying screen.

**RESULTS**

The technique of Eckhardt (6) was first tested with *A. tumefaciens C58* which is known to carry two plasmids, the 208-kan toplasid pTiC58 (7) and a 436-kbp cryptic pAT-C58 plasmid (Villarreal and Van Montagu, personal communication). Cells were converted to spheroplasts by incubation with lysozyme in the loading well of an agarose gel. Subsequently, a mixture of SDS was layered carefully over the bacterial suspension. At the onset of electrophoresis, the SDS migrated into the bottom layer, lysing the spheroplasts and releasing the DNA and RNA. The RNA was partially degraded by the RNase in the sample buffer. Prolonged electrophoresis caused CCC of DNA and fragmented linear DNA to migrate into the gel. Figure 1A, lane 2, shows the electropherogram of *A. tumefaciens C58* cells after staining with EtBr. The large amount of fluorescence at the gel origin represents most of the bacterial DNA and may include some nicked circular DNA. A broad band is visible near the bottom of the gel, presumably representing heterogeneous fragments of bacterial DNA. In between these two bands of chromosomal DNA two sharp bands of the two plasmids are visible.

To adapt this gel technique to eucaryotic cells, we used cells that are known to carry extrachromosomal CCC of DNA. When the identical conditions of Eckhardt were used, we did not observe circular viral DNA in Raji Burkitt lymphoma cells which carry 50 circular EBV genomes (3). Therefore, we modified the conditions. The final modifications were: omitting lysozyme, including 1 mg of pronase per ml in the lysis buffer, lowering the temperature of electrophoresis to 4°C, limiting the amount of cells to 1 × 10⁶ per well in vertical gels, or running the gels horizontally to accommodate up to 2.5 × 10⁶ cells per well. In the same gel as that used for *A. tumefaciens*, suspensions of three mammalian cells were subjected to the modified condition. Figure 1A, lane 1, shows the DNA pattern of 70N2 cells, a nonproducer cell.
line carrying ca. 200 circular *H. saimiri* genomes of 184 kb per cell (28). In the EtBr-stained gel (Fig. 1A, lane 1), a band is detected migrating slightly faster than the 208-kb pTiC58 plasmid shown in lane 2. Since the mobility of plasmids in this region of the gel is inversely proportional to the log of molecular weight, the size of the 70N2 plasmid is estimated to be slightly less than 200 kb. Compared with bacterial cells, 70N2 cells show significantly more DNA migrating within the front of linear DNA fragments near the bottom of the gel. The amount of DNA in this region may vary considerably for eucaryotic cells and has been noted to be more abundant when the cells were obtained from cultures of high density and relatively low viability (less than 85%). The DNA possibly arose, therefore, from the partially degraded chromosomes of nonviable cells.

The DNA pattern of the EBV-producer cell line P3HR-1 is shown in lane 3 of Fig. 1. A faint band migrating at a rate slightly faster than the 208-kb Ti plasmid is detectable by EtBr staining of the gel (Fig. 1A, lane 3). When the DNA is transferred to APT paper and hybridized to the EBV-specific probe pDK-52 (Fig. 1B, lane 3), this band can easily be detected. The size of the circular EBV genome in P3HR-1 cells has been determined by electron microscopy to be 179 kb (11) and would thus be expected to have the approximate electrophoretic mobility as that observed in Fig. 1A and B, lanes 3. P3HR-1 cells contain an average of 11 to 14 circular viral genomes per cell (5).

In addition to the circular forms of EBV DNA in P3HR-1 cells, linear forms have also been detected by density gradient centrifugation (5) at concentrations of several hundred copies per cell, reflecting the moderate level of infectious virus produced by these cells (13). Gel analysis of P3HR-1 cells reveals a discrete, sharp band migrating within the linear chromosomal fragment zone (Fig. 1A, lane 3); this band hybridizes to the EBV-specific probe pDK-52 (Fig. 1B, lane 3). This hybridization is specific for EBV sequences since fragmented chromosomal DNA from either the EBV-negative cell line 8402 (23) (Fig. 1B, lane 4) or nonproducing cells (Fig. 2, lane 4) do not show any hybridization. This result thus suggests that in this gel system molecules of both plasmid and linear viral DNA can be detected.

**Linear viral DNA of producer cell lines.** The EBV DNA found in infectious virus particles is a linear duplex of 160 kb (15). Since cell line P3HR-1 is known to produce virus progeny, it seems likely that the EBV-specific band in the chromosomal fragment zone represents the linear form of viral DNA rather than degraded chromosomal DNA with integrated EBV genomes or degraded circular EBV DNA. To test this interpretation, various EBV producer cell lines were examined (Fig. 2). To reduce the amount of extracellular virus in the sample, cells were washed in PBS before being suspended in buffer A. The results obtained with EBV-producing cell line QIMR-WIL (18) are shown in Fig. 2, lane 1; a significant amount of total DNA is seen in the zone of linear DNA fragments. Although a discrete band cannot be resolved in this region by EtBr staining, transfer of DNA to APT paper followed by hybridization to the EBV-specific probe pBam A reveals a unique band having the same relative mobility as the lower EBV DNA band of P3HR-1 cells shown in Fig. 1. Circular genomes are not detected in the QIMR-WIL cells shown here.

To further characterize the viral DNA at the linear front, we examined Jijoye cells. EBV production in Jijoye cells can be enhanced by shifting the incubation temperature from 37 to 33°C (24). Gel analysis of Jijoye cells cultured for 1 week at 33°C (Fig. 2A and B, lanes 2) was compared with analysis of cells grown continuously at 37°C (Fig. 2A and B, lanes 3). A significant increase in EBV-specific DNA was revealed after the temperature shift.

This increased amount of EBV-specific DNA migrates in the range of partially degraded, linear cell DNA. Since this band increases in intensity under the same conditions as those reported to stimulate virion production (24), it presumably represents the linear form of the viral genome. We also observed that the number of circular genomes per cell is much less affected by the shifting of temperature than by the amount of linear DNA. This suggests that circular viral DNA replication in these cells is regulated by a mechanism different than that involved in linear viral DNA replication.

We also examined P3HR-1 cells, an EBV-producer cell line which, like its parent cell line Jijoye, increases production of infectious virus when the culture temperature is shifted from 37 to 33°C (17). The amount of hybridization of EBV DNA in the region of linear DNA fragments shows a strong increase as a result of the temperature shift from 37 to 33°C. This is seen when we compare lanes 5 (37°C) and 6 (33°C) of Fig. 2A and B. The concentration of circular forms, however, is again not greatly affected by this temperature shift.
Raji cells, a nonproducer cell line (20), contain 50 to 60 copies of the EBV genome per cell in a conformation of CCC (16). The size of these circular molecules has been determined by electron microscopy to be 178 kb (5). Gel analysis of this cell line (Fig. 2A and B, lanes 4) reveals a unique band migrating with the mobility of a plasmid of ca. 180 kb. No hybridization is detected in the band of linear DNA fragments in this lane, consistent with the nonproducing status of Raji cells.

To show that the sharp band visible in the lower part of the gel of EBV-producing cells is indeed linear viral DNA, we compared its migration with that of purified EBV virions and DNA and that of herpes simplex virus type 1 (HSV-1) virions and DNA, all subjected to the same lysis and electrophoretic conditions as were EBV-producing P3HR-1 cells (Fig. 3A). The first four lanes of Fig. 3A and B display EBV DNA, DNA from nonproducer Raji cells, EBV-producer P3HR-1 cells, and virions purified from P3HR-1 cells, respectively. For comparison, Fig. 3A, lanes 5 and 6 contain HSV-1 DNA and virions, respectively. As size markers for smaller DNA, adenovirus type 1 (Ad1) virions are loaded in Fig. 3A, lane 7, whereas lane 8 has a mixture of Ad1 DNA (35 kb) and its EcoRI fragments (27.6 and 3 kb). The DNA in lanes 1 to 4 of Fig. 3 are transferred to nitrocellulose and hybridized to EBV DNA. Figure 3B shows that the bands in Fig. 1A, lane 3, Fig. 2A, and Fig. 3A, lane 3, are indeed EBV DNA and comigrate with EBV DNA in Fig. 3A and B, lane 1; lanes 2 (Raji) and 3 (P3HR-1) of Fig. 3A and B also show circular EBV DNA. The smear of DNA (lane 2) is presumably a result of the higher amount of nonviable Raji cells (25%) than of P3HR-1 cells (10%; lane 3) in this experiment (Fig. 3A and

FIG. 2. Detection of circular and linear EBV DNA in lymphoid cells. (A) Fluorograph of 0.8% agarose gel prepared and developed as described in the legend for Fig. 1A except cells were washed in PBS before being suspended in sample buffer. Lane 1, QIMR-WIL cells, an EBV-producer cell line; lane 2, Jijoye cells, an EBV-producer cell line grown at 33°C; lane 3, Jijoye cells grown at 37°C; lane 4, Raji cells, a nonproducer EBV cell line; lane 5, P3HR-1 cells grown at 37°C; lane 6, P3HR-1 cells grown at 33°C. (B) Autoradiograph of the same gel after transfer to APT paper and hybridization to a 32P-labeled plasmid containing EBV BamHI fragment A. All conditions are as described in the legend for Fig. 1B.
were for Fig. 1A. Lane 1, 5-78 cells from an *H. ateltes* 73-induced lymphoma in ACCRB rabbits; cells were maintained in a quiescent state at 37°C; lane 2, 22CM37 cells (cottontopped marmoset lymphocytes transformed in vitro by *H. ateltes*); cells had been stored in glycerol-dimethyl sulfoxide at −70°C for 2 years; lane 3, 1670 cells; and lane 4, 70N2 cells, both 1670 and 70N2 cells were maintained in a quiescent state at 37°C. Lane 2, 22CM37 cells (cottontopped marmoset lymphocytes transformed in vitro by *H. ateltes*); cells had been stored in glycerol-dimethyl sulfoxide at −70°C for 2 years. Lane 3, 1670 cells. Lane 4, 70N2 cells. Both 1670 and 70N2 for Fig. 1B. (C) Autoradiogram of the same APT paper as that shown in panel B. After completion of the experiment shown in panel B, the *H. ateltes* probe was eluted with 0.5 N NaOH, and the paper was prehybridized and hybridized with 32P-labeled pSP47 containing the left-most 15.1 kb of *H. saimiri* 11 L-DNA under the conditions described in the legend for Fig. 1B.  

**FIG. 4.** Detection of circular genomes in cell lines from *H. ateltes*- and *H. saimiri*-induced tumors. (A) Fluorogram of an 0.8% agarose gel prepared and developed as described in the legend for Fig. 1A. Lane 1, 5-78 cells from an *H. ateltes* 73-induced lymphoma in ACCRB rabbits; cells were maintained in a quiescent state at 37°C; lane 2, 22CM37 cells (cottontopped marmoset lymphocytes transformed in vitro by *H. ateltes*); cells had been stored in glycerol-dimethyl sulfoxide at −70°C for 2 years; lane 3, 1670 cells; and lane 4, 70N2 cells, both 1670 and 70N2 cells were maintained in a quiescent state at 37°C. Lane 2, 22CM37 cells (cottontopped marmoset lymphocytes transformed in vitro by *H. ateltes*); cells had been stored in glycerol-dimethyl sulfoxide at −70°C for 2 years. Lane 3, 1670 cells. Lane 4, 70N2 cells. Both 1670 and 70N2 for Fig. 1B. (C) Autoradiogram of the same APT paper as that shown in panel B. After completion of the experiment shown in panel B, the *H. ateltes* probe was eluted with 0.5 N NaOH, and the paper was prehybridized and hybridized with 32P-labeled pSP47 containing the left-most 15.1 kb of *H. saimiri* 11 L-DNA under the conditions described in the legend for Fig. 1B.

The results clearly show that the sharp band observed in EBV-producer cells (Fig. 1, 2, and 3) comigrates with EBV DNA purified (Fig. 3B, lane 1) or released from virions (Fig. 3, lanes 4) and with HSV-1 DNA and virions (Fig. 3A, lanes 5 and 6, respectively) and migrates slightly slower than does Ad1 DNA from virions (Fig. 3A, lane 7) or purified Ad1 DNA and its EcoRI fragment A (Fig. 3A, lane 8). Smaller linear DNA migrates faster as the Ad1 EcoRI fragment B in Fig. 3A, lane 8, shows; EcoRI-C has migrated off the gel. However, the gel system does not discriminate well between linear DNA in the 27-kb (Ad1 EcoRI-A) and 160-kb (herpesvirus DNA) range.

**H. saimiri and H. ateltes.** The intracellular forms of viral DNA found in lymphoma cell lines carrying *H. saimiri* and *H. ateltes* genomes are shown (Fig. 4); cell line 5-78, derived from an *H. ateltes*-induced lymphoma of ACCRB rabbits, reveals two distinct bands migrating as CCC of DNA of ca. 190 and 180 kb (lane 1). Although differences in genetic content of these two plasmids have not been determined, each plasmid is seen to hybridize to *H. ateltes* DNA isolated from infectious virus particles (Fig. 4B, lane 1). It is interesting to note that there is only a very small amount of fragmented chromosomal DNA released from 5-78 cells. Before gel analysis these cells were cultured for 7 days at a low cell density (ca. 104 cells per ml), which did not stimulate growth. Such a resting culture may not contain many nonviable cells, which presumably give rise to degraded chromosomes. Analysis of a sample of these cells from an exponentially growing culture revealed a more typical amount of DNA in this region (data not shown). In addition, no linear *H. ateltes* DNA can be detected from these cells by hybridization, as expected of nonproducer cells. Cell line 22CM37, derived in vitro from *H. ateltes*-transformed marmoset lymphocytes is shown (Fig. 4, lanes 2). In the autoradiogram shown, *H. ateltes* CCC of DNA cannot be detected; however, long exposures of a separate analysis of these cells (data not shown) revealed weak hybridization to a band in the area of CCC of DNA of ca. 190 kb. 22CM37 cells used in the experiment shown here came from a vial frozen for ca. 2 years in glycerol and dimethyl sulfoxide at −70°C. Subsequent to this experiment, we compared 70N2 cells frozen for ca. 2 years at −70°C and actively growing cells (data not shown); in the frozen cells, we could barely detect hybridization of CCC DNA in the range of 184 kb, but the lane with fresh 70N2 cells revealed the normal amount of hybridization. Raji cells, however, stored for 1 week in loading buffer at −70°C yielded gel patterns identical to those of actively growing cells.

**H. saimiri** cell lines 1670 and 70N2 are shown in Fig. 4, lanes 3 and 4, respectively. The viral DNA in both of these cell lines has been shown by electron microscopy and CsCl-EtBr density gradient centrifugation to exist in a CCC conformation. The size of *H. saimiri* DNA in 1670 cells has been determined to be 209 kb and in 70N2 cells is 184 kb (28). A band corresponding to plasmids of these sizes can be detected by EtBr staining of the gel (Fig. 4A). Subsequent to the *H. ateltes* hybridization analysis shown in Fig. 4B, the same APT paper was washed under denaturing conditions and then hybridized to plasmid pSP47 containing *H. saimiri* specific DNA sequences. The autoradiogram obtained is shown in Fig. 4C. Both cell lines show specific hybridization to this probe in the area of 190-kb CCC, whereas no linear viral DNA forms are detected. This correlates with the reported nonproducer status of these cell lines (28).
4.5 V/cm), to hybridized to nick-translated EBV pDK-52 plasmid. Wells 1 through 5 contain 2.5 x 10^6 8402 cells plus; (lane 1) 5 x 10^6 Raji cells; (lane 2) 2.5 x 10^6 Raji cells; (lane 3) 1.25 x 10^6 Raji cells; (lane 4) 2 x 10^5 P3HR-1 cells; and (lane 5) 1 x 10^5 P3HR-1 cells. Controls were (lane 6) 10^6 Raji cells and (lane 7) 10^6 P3HR-1 cells.

DISCUSSION

CCC forms of DNA can be detected in mammalian cells by gentle lysis of the cells in the well of an agarose gel followed directly by electrophoresis. This procedure, originally described by Eckhardt (6) for detecting bacterial plasmids, resolves plasmids ranging in size from 3.2 to 600 kb (22). The method of Eckhardt is easily modified for mammalian cells as shown in this paper. These modifications include: electrophoresis at 4°C to reduce nuclease activity and to prevent excessive heating of the gel, the addition of pronase to the lysing mixture (probably removes tightly bound nuclear proteins), application of fewer total cells to each lane of the gel, and the omission of lysozyme from the lysis mixture. The procedure is rapid and requires only 5 x 10^5 cells. The limit of detection of CCC of DNA in the vertical gel system is two to five viral genomes per cell. Adapting the method to horizontal gels resulted in an increased sensitivity of at least 0.25 circular EBV genome per cell (Fig. 5).

We conclude that this gel system, in addition to detecting circular DNA forms, can also be used to detect linear 160-kb DNA. We base this conclusion on the following results: (i) a sharp band can be seen in fluorographs below the major area in which partially degraded chromosomal DNA migrates when EBV producer cells are analyzed but not when nonproducer cells (e.g., Raji) are tested, (ii) this band increases sharply in intensity under conditions in which virus production is known to increase, e.g., a shift in temperature from 37 to 33°C for P3HR-1 cells (Fig. 2, lanes 5 and 6) and Jijoye cells (Fig. 2, lanes 2 and 3), (iii) hybridization of EBV DNA increases seemingly proportional to the increase in fluorescence of this band. (iv) the amount of hybridization of EBV DNA to this band is directly correlated with conditions known to increase the amount of virus production and is independent of the amount of cellular DNA degradation (cf. Fig. 2A and B, lanes 5 and 6), (v) HeLa cells infected with HSV-1 show a similar band in this area which hybridizes to a cloned HSV-1 DNA fragment (data not shown), but this hybridization is not detected in uninfected control HeLa cells, (vi) purified P3HR-1 virions or EBV DNA show DNA comigrating with this band which hybridizes to EBV DNA, (vii) this band also comigrates with HSV-1 DNA and the DNA released from HSV-1 virions, (viii) Ad1 DNA (35 kb) either purified or released from virions migrates slightly faster than does EBV DNA (160 kb). Under the conditions used, Ad1 EcoRI-A (28 kb) migrates only slightly faster than does Ad1 DNA (35 kb), whereas EcoRI-B (5.6 kb) migrates considerably faster than does EcoRI-A (Fig. 3A, lane 8).

As is expected (1), large linear EBV DNA migrates considerably faster than does its equally sized circular DNA. Small circular DNA such as that of pBR322 or simian virus 40 comigrates with the band of broken chromosomal DNA (data not shown).

We have found the gel technique to be useful in examining the intracellular state of herpesvirus genomes in immortalized cell lines. In the lymphoblastoid cell lines studied (except perhaps QIMR-WIL), the circular form is always present, whereas the linear DNA form may or may not be
detected. Thus, the circular viral genome is indicative of latent, nonproductive infection, whereas the existence of linear forms indicates virus-producing cells. The role of the viral genome in such transformed cell lines and the significance of its physical conformation are not well understood.

The simple procedure used here, however, may aid in the study of such cell-virus relationships by allowing the separation and detection of small amounts of circular viral genomes in the presence of large excess of linear forms in virus-producing cells.

This gel technique has also been used to probe for the presence of EBV genomes in cells of patients with X-linked lymphoproliferative disease, an immunodeficiency syndrome (12). Cell lines from two patients who had not responded to antiviral therapy were found to carry circular but no linear EBV genomes (27). We are continuing this study to investigate the possible correlation between the presence of circular viral genomes and resistance against antiviral therapy.

We expect the method to be useful for other DNA analyses in mammalian cells as well. For example, preliminary results show that human mitochondrial DNA, which is known to be a molecule of CCC, can easily be detected by hybridization to recombinant clones containing human mitochondrial DNA sequences (gifts of Bruce Roe; unpublished data). The technique would be useful for the detection and characterization of extrachromosomal DNA arising as a result of (i) viral infection, (ii) DNA transfer experiments, and perhaps even (iii) intermediates in translocatable elements and chromosome rearrangements.

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LITERATURE CITED


