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Epstein-Barr Virus DNA Is Amplified in Transformed Lymphocytes

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Leukocytes isolated from two adult donors who lacked detectable antibodies to antigens associated with Epstein-Barr virus were exposed to an average of 0.02 to 0.1 DNA-containing particles of Epstein-Barr virus per cell and immediately cloned in agarose. Within about 30 generations all transformed cell clones contained between 5 and 800 copies of viral DNA per cell. Only 1 in 10^4 to less than 1 in 10^5 of the cells of each clone released virus, and the frequency of release did not correlate with the average number of copies of viral DNA in the cells of each clone. One clone that had an average of five copies of viral DNA per cell was recloned, and the average number of copies in four of six subclones increased 15- to 50-fold while the subclones were being propagated sufficiently to study them. These results indicate that Epstein-Barr virus DNA can undergo amplification relative to cell DNA at different times after it transforms cells.

Tumor cells from biopsies of patients with Burkitt's lymphoma and nasopharyngeal carcinoma contain many copies of Epstein-Barr viral (EBV) DNA (6, 8, 18). Similarly, all lymphoid cell lines established from tumor biopsies from patients with diseases unrelated to EBV and from healthy donors contain, on the average, many copies of EBV DNA per cell (18). Most of these cell lines contain few if any cells that express viral capsid antigens, indicating that the cells release little if any virus. The bulk of the intracellular viral DNA molecules in those cell lines studied are full length, unintegrated, and circular (7, 11) and contain most, if not all, of the nucleotide sequences found in extracellular virus (14).

Two disparate mechanisms could give rise to these multiple copies of viral DNA in transformed lymphocytes. Either each cell could be infected by many particles of EBV, each one contributing one copy of viral DNA, or each cell could be infected by only one particle of EBV and its viral DNA could be amplified during the growth of the transformed cell. It is not practical to test whether the former mechanism operates in vivo. To test whether the latter mechanism can operate in vitro, adult leukocytes were exposed to 0.02 to 0.1 particles of EBV per cell, and then the cells were cloned immediately in agarose by using the conditions established for a clonal transformation assay (15). When the clones had grown sufficiently to be tested (approximately 25 to 30 cell divisions), all had on the average multiple copies of viral DNA per cell. One clone with an average of five copies of EBV DNA per cell was recloned, and in four subclones the average number of copies of viral DNA per cell had increased 15- to 50-fold by the time the cells could be assayed. These findings indicate that EBV DNA is amplified in at least some of the cells it transforms.

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MATERIALS AND METHODS

Cells and virus. A clone was selected from the B95-8 marmoset cell line (9) and was grown in suspension in RPMI 1640 medium containing 5% calf serum plus antibiotics. The supernatant of these cells after low-speed centrifugation and passage through a 0.22 μm filter was used as a source of EBV. Peripheral lymphocytes from EBV-seronegative donors were purified and clonally transformed as described previously (15).

Detection of cells that release EBV. To detect virus release, cells were irradiated with 4,000 rads of γ-irradiation and then cocultivated with a 25- to 50-fold excess of seronegative human lymphocytes for 24 h. All of the cells were then cloned in agarose over an irradiated feeder layer as described previously (15). When this procedure was used, for each B95-8 cell that stained positively with antisera to viral capsid antigens, 0.5 to 2 clones were observed growing after 4 to 6 weeks in culture.

Purification and detection of viral DNA. EBV DNA was purified from partially purified extracellular virus. The B95-8 cell supernatants were clarified by low-speed centrifugation and passage through a membrane prefilter (catalog no. AP1514250; Millipore Corp.). Virus was precipitated from this solution with polyethylene glycol as described by Adams (1). The

virus pellet was dissolved in 1/50 of the original volume in phosphate-buffered saline plus 0.01 M $MgCl_2$. This solution was treated with 10 μg of DNase per ml for 2 h at 37°C. $MgCl_2$ was chelated by bringing the solution to 0.05 M EDTA, and the virus was pelleted through a 10-ml cushion of 20% Renografin (Squibb) by centrifuging the solution at 25,000 rpm for 1 h. The pellet was resuspended in 0.1 M EDTA, and the solution was brought to 1% Sarkosyl and 1 mg of self-digested pronase per ml and incubated at 37°C for 4 h. CsCl was added to this solution to give a refractive index of 1.402, and the solution was centrifuged at 33,000 rpm in an angle rotor for 60 h at room temperature. Fractions containing EBV DNA were pooled, a solution of CsCl (refractive index, 1.401) was added, and the DNA was sedimented to equilibrium again. The DNA was collected, dialyzed, and sedimented at 10,000 rpm in a Spinco SW27 rotor for 46 h at room temperature in a gradient of 10 to 30% sucrose containing 1.0 M NaCl, 0.01 M Tris-hydrochloride, pH 8.0, and 0.001 M EDTA. The EBV DNA which sedimented just slower than T4 DNA was pooled, dialyzed, and used for all experiments.

Intracellular EBV DNA was detected by using renaturation kinetics as our assay. Cell DNA was purified by the method of Botchan et al. (2), EBV DNA was labeled with "nick translation" by using [^{3}H]thymidine triphosphate or [^{125}I]deoxycytidine triphosphate (13), and DNA-DNA renaturation was performed in solution as described previously (16).

RESULTS

The general design of our experiments is shown in Fig. 1. First, the number of copies of virion DNA in a cell-free stock of EBV was determined by using renaturation kinetics to measure the concentration of DNase-resistant EBV DNA (16). We assumed that each particle of EBV contained no more than one copy of viral DNA. Two transformation experiments

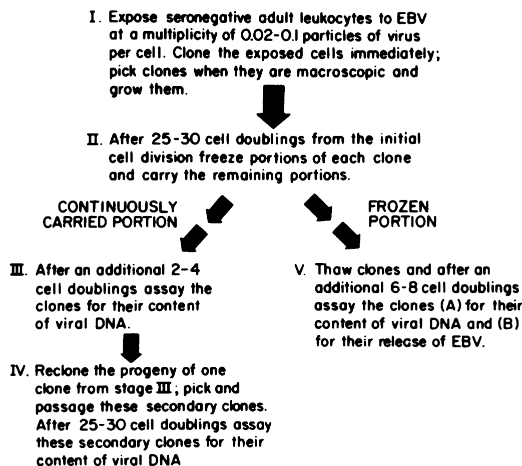

FIG. 1. *Experimental design of the generation and study of clones of lymphocytes transformed by one DNA-containing particle of EBV.*

were done with this stock of virus. Peripheral leukocytes from one EBV-seronegative adult donor were exposed to 0.05 to 0.1 DNA-containing particles of EBV per cell, and the cells were cloned in agarose (15). Eight separated clones were picked and propagated from this experiment and are designated by the prefix 11/17-. In the second experiment peripheral leukocytes from a different EBV-seronegative adult donor were exposed to half as many DNA-containing particles (0.02 to 0.05) and cloned similarly. Nine separated clones were picked and propagated from this experiment and are designated by the prefix 3/15-.

The doubling times of the clones varied from 40 to 120 h so that the experiments studying them were carried out asynchronously. The number of times each clone had doubled when it was tested was estimated by the logarithm of the number of cells that had accumulated for that clone. This number was necessarily imprecise because not all of the cells of a clone could be recovered from the agarose and not all of the cells in a clonal population were viable. Viable portions of all 17 clones were frozen at about cell division 25 to 30.

Samples of seven clones were thawed, grown for six to eight cell divisions, and assayed for their content of viral DNA and for their ability to release infectious particles. Figure 2 presents the results from an experiment in which renaturation kinetics was used to measure the EBV DNA concentration in each cell population. These data also show that the purified, in vitro-labeled viral probe used did not contain detectable human DNA sequences (Fig. 2A). The average number of copies of viral DNA found per cell could be calculated by knowing the amount of cell DNA tested, the data in Fig. 2, and that the cells are diploid (Table 1). We found that all clones had on the average more than a single copy of viral DNA per cell and that the number ranged from 10 to 750 copies per cell.

One of the clones (3/15-9) was passaged for an additional 30 to 40 cell divisions and tested for its content of viral DNA. Both the cells grown for about 30 to 35 cell divisions (3/15-9N) and those grown for about 60 to 70 cell divisions (3/15-9-0) contained on the average 200 copies of EBV DNA per cell (Table 1).

When the thawed clones were tested for their content of viral DNA, they were assayed simultaneously for their release of infectious EBV. No virus was detected in the cell supernatants of these clones, as monitored by a transformation assay. However, when the clones were γ-irradiated and cocultivated with a 25- to 50-fold excess of seronegative leukocytes, some transformed colonies did grow out, indicating that the major-

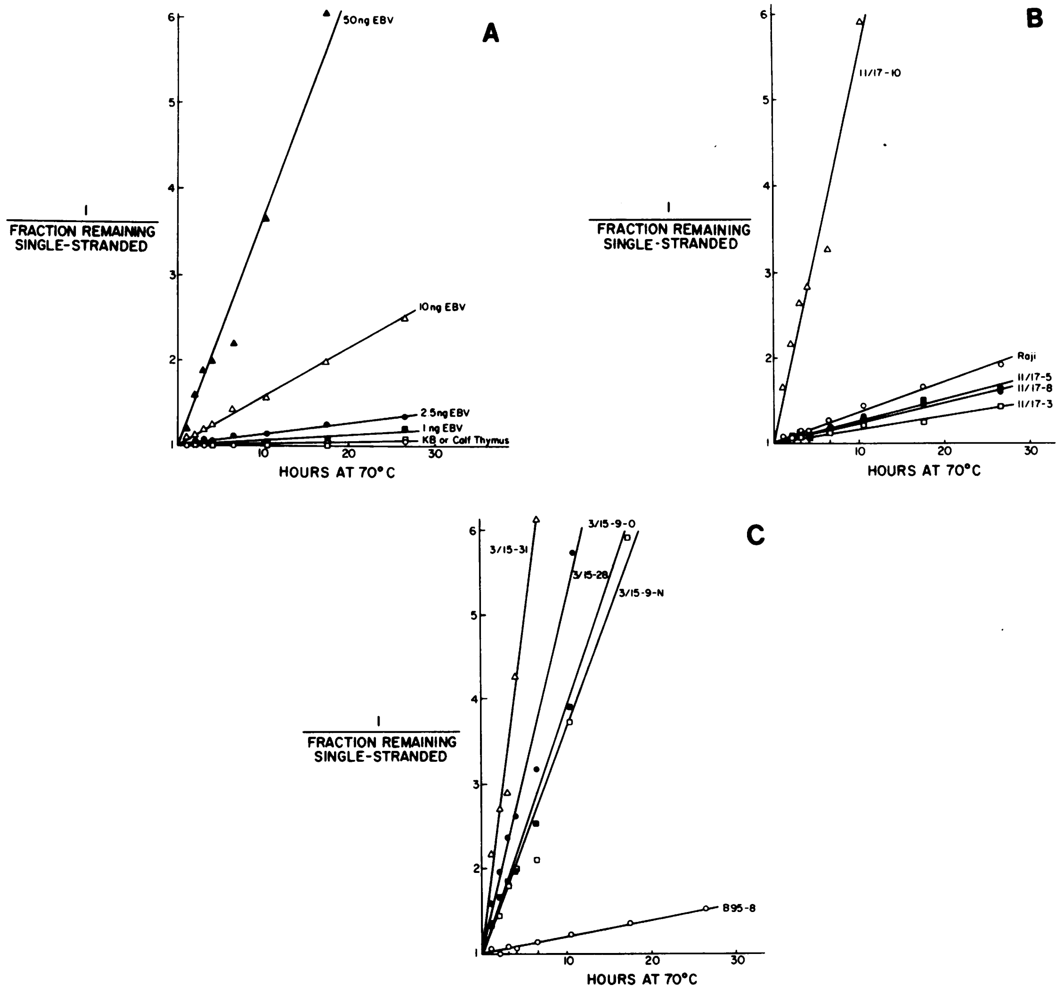

FIG. 2. *Detection of EBV DNA by renaturation kinetics. Data from the different clones are designated by 11/17- or 3/15- to indicate from which transformation experiment the clone arose and by a succeeding number to define each clone. Cell DNAs or purified viral DNA were sheared, denatured, and mixed with denatured, in vitro-labeled EBV DNA. The solutions were all brought to the same concentration of DNA by adding calf thymus DNA where necessary. The solutions were incubated at 70°C, and the extent of renaturation of the labeled probe was determined by chromatography on hydroxylapatite columns. Each point represents 1,500 to 2,000 cpm of labeled probe. The slope of each plotted line was determined by linear regression analysis. (A) Reconstruction experiments which indicate the sensitivity of the assay (1 ng of EBV DNA can be detected) and show that human cell DNA (KB cells) does not accelerate the renaturation of the viral DNA more than the negative control (calf thymus DNA). To determine the quantity of EBV DNA per cell, we determined the amount of viral DNA in a sample of cell DNA by comparing its slope with that of the reconstruction experiments and then corrected for the number of cell equivalents in the cell DNA sample. The data represented in this figure are analyzed in Table 1.*

ity of clones did release virus at a low level (Table 1). The amount of detectable EBV released from each clone did not correlate with the average number of copies of viral DNA per cell found in each clone.

All of the clones from continuously growing stocks (never frozen) were also assayed for their content of viral DNA. These cells, grown for about 27 to 34 cell divisions from the original transformation event, contained multiple copies of viral DNA. The number of copies of viral DNA per cell ranged from 5 to 800 (Table 2). The number of viral DNA molecules per cell for those clones that were tested in each set of experiments was the same within experimental error (Tables 1 and 2). This finding indicates

TABLE 1. *Measurement of viral DNA and release of virus from clones*

Clone	Amt of cell DNA tested (μg)	Amt of EBV DNA found in cell DNA (ng)[a]	Avg no. of EBV DNA copies per cell[b]	No. of cells releasing virus per 10^5 cells[c]
Raji[d]	9	6	25	<3
B95-8[d]	0.4	4	400	4,000
11/17-3	13	3	10	3
11/17-5	10	5	20	<3
11/17-8	9	4	20	<3
11/17-10	10	100	400	3
3/15-9-N	11	50	200	<1
3/15-9-0	11	50	200	5
3/15-28	9	160	700	5
3/15-31	10	190	750	0.5

[a] The 0.2 ng of probe DNA has not been subtracted from these values. These values are taken from the slopes of the curves shown in Fig. 2.

[b] Based on a molecular weight of human cell DNA of 4×10^{12}.

[c] This number was obtained by cocultivating γ-irradiated cells for 24 h with a 50-fold excess of seronegative leukocytes and then cloning the cells in agarose. The values were derived from a standard curve using B95-8 cells, where for each two viral capsid antigen-positive cells cocultivated with leukocytes, one clone grew. The values with a < sign indicate that no clones were observed and give the limit of detection for the experiment.

[d] Raji is a cell line established from a Burkitt's lymphoma biopsy by Pulvertaft (12), and B95-8 is the transformed marmoset cell line established by Miller and Lipman (9) from which we harvested EBV.

that freezing cells for viable storage and differences in lifespan of a few cell generations do not alter the average content of viral DNA.

One cell clone (11/17-5; Table 2) was recloned; six subclones were picked and propagated (labeled rec in Table 2). The original clone had on the average five copies of viral DNA per cell, which is as small a number as we found. Clonal populations of its progeny after the 25 to 30 cell divisions required to perform the experiment had varying numbers of copies of EBV DNA per cell. Two of the six subclones had numbers comparable to that of the parent, 11/17-5, whereas the other four had further amplified their viral DNA to levels between 80 and 250 copies per cell (Table 2).

DISCUSSION

Peripheral leukocytes from two EBV-seronegative donors were exposed to 0.02 to 0.1 DNA-containing particles of EBV per cell, and all of the resulting clones of virally transformed cells were found to contain multiple copies of viral DNA. We believe that it is likely that because of the low ratio of virus to cells most, if not all, transformed cells resulted from infection of one cell by a single EBV particle. Only 1 in 30 to 1 in 50 DNA-containing particles in our stocks of EBV is capable of transforming cells, between 3 and 10% of peripheral leukocytes are capable of being transformed, and one particle is sufficient to transform a cell (15). It seems likely, therefore, that each cell capable of being transformed in the target population was exposed, on the average, to less than one "infectious" particle of EBV. Two different ratios of DNA-containing particles to cells were used to generate the two sets of transformed clones (0.05:1 to 0.1:1 for the 11/17 series and 0.02:1 to 0.05:1 for the 3/15 series). The average content of viral DNA in the different transformed clones was independent of the ratio of DNA-containing particles to cells

TABLE 2. *Measurement of viral DNA in clones and subclones*

Clone designation	Amt of cell DNA tested (μg)	Slope of linear regression	Amt of EBV DNA found in cell DNA (ng)[a]	Avg no. of EBV DNA copies per cell[b]
11/17-1	40	1.2×10^{-1c}	170	170
11/17-2	125	4.1×10^{-2c}	50	15
11/17-3	225	5.4×10^{-2c}	70	10
11/17-4	55	1.6×10^{-1c}	230	170
11/17-5	850	1.9×10^{-1d}	125	5
11/17-8	90	4.4×10^{-2d}	20	10
11/17-9	90	9.4×10^{-2d}	55	25
11/17-10	15	1.5×10^{-1d}	100	250
3/15-1	180	6.2×10^{-1c}	900	200
3/15-9	20	1.4×10^{-1d}	90	180
3/15-25	180	3.8×10^{-1c}	550	120
3/15-28	8	2.4×10^{-1d}	160	800
3/15-29	150	3.2×10^{-1c}	470	125
3/15-31	10	1.7×10^{-1d}	110	450
3/15-34	200	2.6×10^{-2c}	30	5
3/15-36	150	3.8×10^{-1c}	550	150
3/15-38	90	2.9×10^{-2d}	10	5
rec C1	25	1.1×10^{-1d}	70	110
rec C4	200	9.8×10^{-2d}	60	10
rec D1	150	1.2×10^{-1d}	75	20
rec D2	20	7×10^{-2d}	40	80
rec D4	20	1.2×10^{-1d}	75	150
rec D5	20	1.8×10^{-1d}	120	250

[a] The 10-ng EBV probe has been subtracted from these values.

[b] Based on molecular weight of human cell DNA of 4×10^{12}.

[c] In these determinations, 62.5 ng of added EBV gave a slope of 4.2×10^{-2}.

[d] In these determinations, 100 ng of added EBV gave a slope of 1.4×10^{-1}.

used to generate them. This ratio was twofold higher for the 11/17 series than for the 3/15 series, but the average numbers of viral DNA copies per cell of the 11/17 clones were not higher than those of the 3/15 clones (Table 1 and 2). This observation is consistent with the assumption that each susceptible cell received at most one particle of EBV and, therefore, the multiple copies of viral DNA found in all the different clones are likely to result from preferential amplification of the viral DNA.

That EBV DNA is amplified in transformed cells is proven by the experiment in which four of six subclones of clone 11/17-5 which contained an average of five copies of viral DNA were found to have amplified their EBV DNA 15- to 50-fold. This last experiment also demonstrates that preferential amplication of viral DNA not only may take place early after transformation but also does take place sometime between the 30th and 60th cell generation after transformation.

Some transformed clones harbor on the average as many copies of viral DNA as does the B95-8 cell line (9), the marmoset cell line used to produce the virus stocks. However, the in vitro-transformed human cell lines release 10^3- to 10^4-fold less virus than do the marmoset cells (Table 1). This finding underscores the general observation that the host cell transformed by EBV does play some role in controlling the expression of its resident viral information. That most clones of transformed cells can release virus, although inefficiently, indicates that EBV-transformed lymphoid cells may be a source of virus during the course of human infection (3, 10).

In other experiments we have studied the intracellular viral DNA in clones 11/17-2 and 3/15-31 (C. R. Kintner and B. Sugden, Cell, in press) and clones 11/17-1, 11/17-4, 11/17-5, 3/15-4, and 3/15-25 (unpublished data). The termini of the virion DNA of the B95-8 strain of EBV are heterogeneous (4) in that different molecules have different numbers of units of a direct, terminal repetition (Kintner and Sugden, in press). Analysis of intracellular viral DNA digested with different restriction endonucleases in the above clones has shown that no fragments identical with those containing the termini of linear virion DNA are present in the clones and each clone has one new viral fragment composed of the two terminal regions found in the linear virion DNA. The absence of linear, viral DNA in the clones excludes the possibility that the amplification of viral DNA has occurred only in a few cells to yield mature, linear virion DNA which for some unknown reason is noninfectious and therefore undetected in our cocultivation assay. The new fragment of digested intracellular, viral DNA which arose from the joining of the termini of virion DNA has a different molecular weight in at least five of the clones studied (Kintner and Sugden, in press; unpublished data). This finding indicates that only one particle of EBV infected each of these clones and that each viral DNA molecule infecting its clone contained a distinct number of the terminal repetitions and/or used a distinct number of those replications during its circularization.

Several features of the viral and cellular DNA in EBV-transformed cells have been established. First, the viral DNA in these cells probably contains most or all of the nucleotide sequences found in extracellular virus. This assertion is based both on the previous findings that the number and size of DNA fragments generated by cleavage with restriction endonucleases of intracellular and extracellular viral DNAs are similar but not identical (14) and on the findings (Table 1) that most of the clones release infectious virus particles, albeit at a low level. Second, cells transformed by EBV in vitro (15) and lines established from Burkitt's lymphoma biopsies (17) remain diploid or near diploid. This latter observation indicates that the amplification of viral DNA does not result from an induced polyploidy of the transformed genome of the cell. Third, although the distribution of viral DNA among the cells in any population is not known, the average number of copies of viral DNA per cell for different clones does not correlate with the frequency of release of infectious virus (Table 1). This finding indicates that the high number of copies of EBV DNA found on the average per cell does not arise from clonal populations containing many cells that are lytic virus factories. Fourth, Nonoyama and Pagano (11) and Lindahl et al. (7) have shown that the bulk of viral DNA in a cell line established from a Burkitt's lymphoma biopsy is present as extrachromosomal, covalently closed molecules of close to full-length DNA. Similar studies on EBV-transformed cell lines established from normal donors have found that the bulk of viral DNA is present in an episomal form (5). The replication of extrachromosomal, viral DNA does not need to be physically or temporally linked to that of the cellular DNA. We suggest that EBV DNA is an independent replicon in the cells that this virus transforms.

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