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Nucleic acid spot hybridization: Rapid quantitative screening of lymphoid cell lines for Epstein–Barr viral DNA

(nucleic acid hybridization/Epstein–Barr virus/lymphocytes)

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ABSTRACT **A simple nucleic acid hybridization method to screen numerous samples of eukaryotic cells rapidly for their Epstein–Barr virus (EBV) DNA content is described. Whole cells are spotted on nitrocellulose filters and their DNA is denatured and fixed to the filter. The resultant DNA spots are hybridized to nick-translated EBV DNA and the extent of hybridization is monitored by autoradiography and scintillation counting. Statistical analysis of serial dilutions of cells permits their viral genome content to be estimated quantitatively by reference to a known standard, such as Raji cells or an artificial mixture of pure viral DNA and uninfected lymphocytes. The sensitivity of the method is between 5 and 50 pg of viral DNA. With this method we are able to select subclones that are high producers of EBV DNA and to identify the optimal time for harvest of EBV DNA from cultured cells. Spot hybridization should permit any cell population or fluid to be screened for the presence of a DNA sequence for which a radioisotopically labeled probe is available.**

Much available information about Epstein–Barr virus (EBV), a human lymphotropic herpes virus, has been obtained with nucleic acid hybridization methods (1). Multiple copies of the viral genome have been found in every cell of nearly all B lymphocyte lines and in biopsy samples from patients with anaplastic nasopharyngeal carcinoma, Burkitt lymphoma, or certain cases of acute malignant lymphoproliferative disease (2–4). Most of the genomes are circular nonintegrated episomes (5). Analysis of Southern blots has shown that, although the cells usually harbor a complete copy of the viral genome, only certain regions are regularly transcribed (6–7).

The techniques of membrane filter hybridization, DNA reassociation kinetics, and hybridization of Southern blots have been applied to relatively few samples because of several constraints. Large amounts of sample cellular DNA are required for each test, 10 μg for cRNA·DNA filter hybridization and 500 μg for reassociation kinetics. The sample DNA must first be purified and sometimes enriched for viral sequences.

A variety of laboratory and epidemiologic studies would be possible with a method that permits rapid quantitative screening of large numbers of samples for viral DNA without the necessity for first purifying viral or cellular DNA. We have found that the colony hybridization method (8), which was developed to screen bacterial clones for a specific DNA sequence, can be employed as a quantitative method for determining the viral genome content of eukaryotic cells.

MATERIALS AND METHODS

Cells. Several different types of continuous lymphoid lines were screened for their content of EBV DNA. These lines, with and without EBV genomes, were derived from patients with Burkitt lymphoma or leukemia, or by EBV immortalization *in vitro* of human lymphocytes from umbilical cord blood (HUCL) or of marmoset lymphocytes. Fresh mononuclear cells were isolated from umbilical cord blood.

Preparation of Filters. Cells were washed once in Hanks' balanced salt solution and resuspended at 10^5–10^6 cells per 5–10 μl of the same solution. Replicate 5-μl samples were applied to a 1-cm^2 square on a nitrocellulose filter. The filter was soaked consecutively in 0.5 M NaOH for 7 min, twice in 0.6 M NaCl/1 M Tris·HCl at pH 6.8 for 1 min, and once in 1.5 M NaCl/0.5 M Tris·HCl at pH 7.4 for 5 min. For these operations the filter was placed spotted-side up on a piece of Whatman 3 MM chromatography paper saturated with the solution and transferred with a forceps. The filter was then dried in air for 20 min, floated onto 95% ethanol, dried in air for 5 min, washed twice in $CHCl_3$, and dried for 15 min. After a final rinse in 0.3 M NaCl, the filter was again dried and was then baked at 80°C for 18 hr.

Nucleic Acid Digestions and Hydrolysis. In some experiments the filter was treated further to learn whether hybridization was due to DNA or RNA. For digestion of DNA the filter was incubated in DNase at 20 μg/ml in 10 mM $MgCl_2$/200 mM NaCl/4 mM KCl/1 mM Na_2HPO_4/2 mM KH_2PO_4 for 4.5 hr at 37°C. It was then washed in 50 mM EDTA/3 × NaCl/Cit (1 × NaCl/Cit = 150 mM NaCl/15 mM sodium citrate) and dried. For digestion of RNA, the filter was incubated for 1 hr at 37°C in RNase at 100 μg/ml in 1 × NaCl/Cit. The RNase had previously been heated at 80°C for 10 min. The filter was then washed three times in 2 × NaCl/Cit and dried. Alkaline hydrolysis was done on cells before they were spotted on the filter by mixing 10 μl of cells with 10 μl of 1 M NaOH and incubating for 3.5 hr at 37°C.

Viral DNA Probes and Hybridization. Viral DNA was isolated from virions released by the B95-8 and FF-41 marmoset lines. We followed described methods (9, 10). ^{32}P-Labeled probes were prepared by "nick translation" (11). Nucleic acid hybridizations contained 1.4 × 10^5–7.2 × 10^6 cpm and were carried out in 6 × NaCl/Cit at 65°C for 48–92 hr (12). Autoradiographs were exposed for about 20 hr as routine.

Analysis of Data. Serial replicate cell dilutions were placed on a filter. One series consisted of Raji cells, which were a reference standard. Another was BJAB cells or primary HUCL, which measured nonspecific binding. The number of EBV genomes in any other line was derived by comparison with Raji cells and the background. The average cpm bound by 10 replicate blank filters was subtracted from cpm bound by each spot, and if this value was negative the sample was deleted from subsequent analysis. A plot of net cpm bound versus cell number was generated by computer, and the computer analyzed the data by least-squares regression. The extent to which variability

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Abbreviations: EBV, Epstein–Barr virus; HUCL, human umbilical cord lymphocytes; NaCl/Cit, 150 mM NaCl/15 mM sodium citrate.

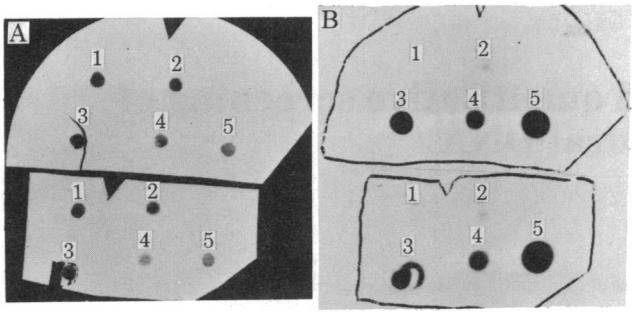

FIG. 1. Spot hybridization detects EBV genomes. (*A*) Five continuous B lymphocyte lines were spotted in duplicate on a nitrocellulose filter. They were: 1, BJAB, a Burkitt lymphoma-derived line that lacks EBV DNA (15). 2, X25-9, a line of neonatal human lymphocytes transformed *in vitro* by EBV that has a low level of EBV genomes per cell (16). 3, FF41-1, a line of marmoset lymphocytes transformed *in vitro* by EBV from saliva. This line is a virus producer. 4, Raji, a Burkitt lymphoma line that contains about 60 genomes per cell (17). 5, HR-1K, a Burkitt lymphoma line that is a producer of nontransforming virus (18). (*B*) The autoradiographic response after hybridization of the filters shown in *A* with 1.4×10^5 cpm of EBV [^{32}P]-DNA.

in the data could be accounted for by a linear model was assessed by calculation of R^2 (13). The slopes of the lines generated by least-squares regression were used for quantification. The slope of the line derived from the EBV-negative cells (HUCL or BJAB) was first subtracted from all other slopes. The average EBV genome number was then calculated by multiplying the ratio of the net slope of the unknown to that of Raji by 60 genomes. Spearman–Karber quantal dose analysis was used to calculate the sensitivity of spot hybridization (14).

RESULTS

Spot Hybridization Detects EBV DNA. In the initial experiment cell spots from five different B lymphocyte lines were placed on two filters. Although the original spots were the same size (Fig. 1*A*), the autoradiographic response after hybridization showed differences that correlated with the content of EBV DNA that these cell lines are believed to contain (Fig. 1*B*). The faintest positive response was seen with the nonproducer X25-9 line, immortalized *in vitro*, and the strongest response with two lines, HR-1 and FF41-1, which are known to have active viral replication. An intermediate reaction was seen in Raji cells, which contain about 60 genomes per cell (17). After 20 hr of exposure to film no hybridization to the cell spot containing the BJAB line, which lacks the EBV genome, was detected on the autoradiograph. However, upon longer exposure to the x-ray film, it was found that the EBV[^{32}P]DNA had bound to the BJAB spots to a minor extent.

We next wished to learn whether hybridization was detecting viral DNA or RNA. Although nitrocellulose filters do not directly bind RNA, basic proteins will bind both to the membrane filters and to RNA, which may thus be trapped (19). Neither alkaline hydrolysis nor RNase digestion reduced the extent of hybridization. DNase digestion, however, nearly totally eliminated specific hybridization to Raji cells and also reduced the background hybridization to HUCL (Fig. 2).

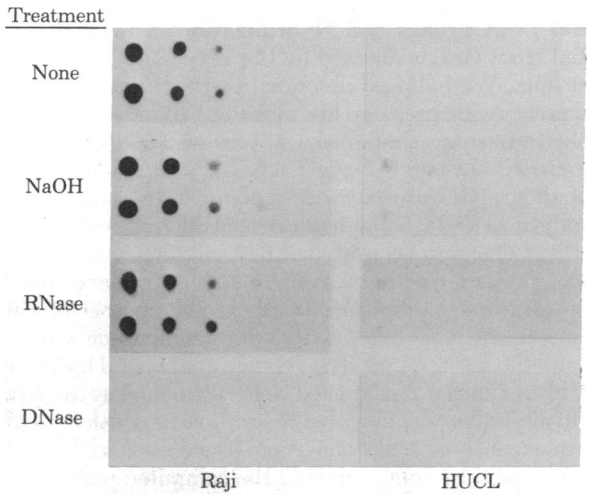

FIG. 2. Effects of alkaline hydrolysis, RNase, and DNase treatments on spot hybridization by Raji and HUCL cells. Starting with 10^5 cells per spot, 1:2 dilutions of cells were placed on replicate filters. The figure is an autoradiogram of the spot hybridizations.

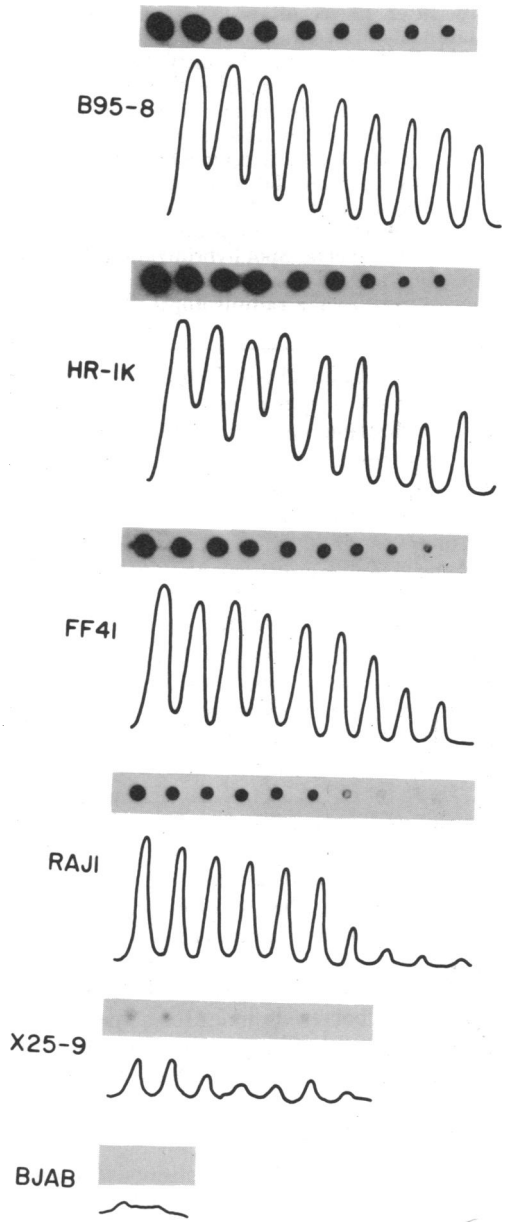

FIG. 3. Attempted quantitation of spot hybridization by densitometer tracing of serial cell dilutions. Serial 1:2 dilutions of cells beginning with 10^6 cells per spot were placed on the filter. The autoradiogram was traced with a Joyce–Loebl densitometer (courtesy of W. C. Summers). B95-8 is a marmoset line that produces transforming virus (20).

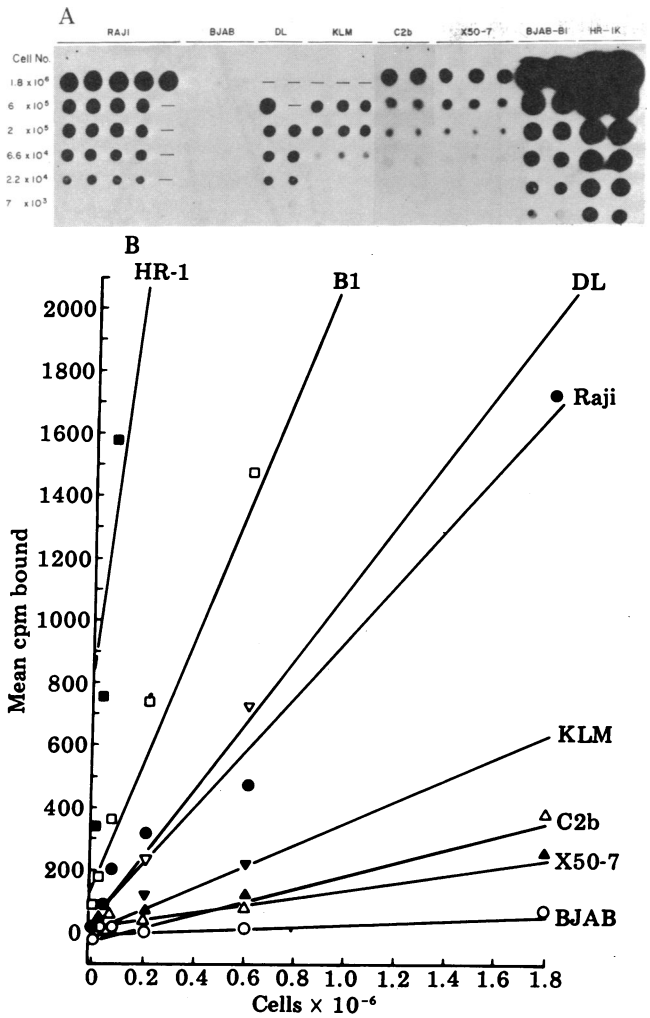

FIG. 4. Quantification of spot hybridization by scintillation counting and computer-assisted analysis. (*A*) Autoradiogram of filter containing serial 1:3 cell dilutions, spotted in replicate, of several different types of B cell lines. DL and KLM are lines established from leukemic patients (21). C2b and X50-7 are HUCL transformed *in vitro* (16). BJAB-B_1 is the BJAB line converted to permanent EBV carriage by superinfection with the P3HR1 strain of EBV (22). (*B*) A redrawn computer-assisted plot of cpm bound versus cell number, for the indicated sources. The lines are plotted with the assumption that the data fit a linear model.

Quantification of Spot Hybridization. Initially we attempted to measure the extent of the spot hybridization reaction by densitometer analysis of the autoradiographs. That there was a quantitative relationship between the number of cells of a particular line in a spot and the intensity of the autoradiograph reaction could be seen in the height of the peaks drawn by the densitometer (Fig. 3). However, this method was not as readily quantified as scintillation counting of individual spots. In a typical experiment (Fig. 4) serial dilutions of various cell lines produced progressively decreasing autoradiographic responses (Fig. 4*A*). A plot of cpm bound versus cell number showed a predominantly linear relationship, although for all cell lines the slope increased more sharply with small numbers of cells (less than 25,000) and in most experiments began to diminish with high numbers of cells (greater than 500,000). The least-squares regression lines for all cell samples tested in this experiment are found in Fig. 4*B*. Data from this and three similar other experiments are compiled in Table 1. Estimates of the genome content per cell of lines known to be producers of virus were high (median 191) and for the nonproducer HUCL lines, immortalized *in vitro*, low (median 5). Least-squares regression analysis showed that for each of the cell lines studied the data fit a linear model (R^2 varied between 0.705 and 0.995).

Table 1. Estimate of EBV genome number and sensitivity of detection by spot hybridization

Cell line		EBV genome equivalents per cell			Sensitivity of detection	
Designation	Type*	No.	R^2†	n‡	CN_{50}§	pg¶
		Experiment 1				
X25-9	A	5	0.705	6	88,400	71
FF41-1	B	191	0.953	7	<2,000	<60
B95-8	B	243	0.854	7	<2,000	<76
HR-1K	C	397	0.729	7	<2,000	<124
Raji	C	—	0.801	7	2,800	27
		Experiment 2				
X25-9	A	5	0.980	7	2,200	2
FF366-3	G	3	0.953	11	4,400	2
FF41	B	72	0.957	42	<800	<9
B95-8	B	84	0.915	44	<800	<11
HR-1K	C	166	0.914	44	<800	<21
Raji	C	—	0.954	42	1,100	11
BJAB	C	0	0.885	38	—	—
BJAB/B_1	D	65	0.891	42	<1,600	<17
Standard	F	28	0.970	40	1,600	7
		Experiment 3				
X50-7	A	6	0.948	18	<7,400	<71
C2b	A	12	0.966	12	7,400	14
HR-1K	C	460	0.976	12	<7,400	<545
Raji	C	—	0.981	26	7,400	71
BJAB/B_1	D	140	0.991	12	<7,400	<166
DL	E	71	0.953	9	<7,400	<84
KLM	E	22	0.926	15	9,300	31
		Experiment 4				
C2b	A	4	0.995	17	<6,200	<4
FF366-3	G	1	0.911	18	17,700	3
Raji	C	—	0.961	22	<781	<8
Standard	F	18	0.901	22	3,100	9

In experiment 1 there was one replicate per cell dilution; in experiment 2, four replicates per cell dilution; in experiments 3 and 4, two replicates per cell dilution.

* The types of cell lines used were: A, nonproducer human lines from umbilical cord blood transformed *in vitro*; B, producer marmoset lines transformed *in vitro*; C, Burkitt lymphoma lines (HR-1K, a virus producer; Raji, a virus nonproducer; BJAB, genome negative); D, the BJAB line converted into permanent EBV carriage by the HR-1K virus (22); E, lines established from leukemic patients (21); F, an artificial standard consisting of a mixture of HR-1 DNA with uninfected HUCL at a ratio of 50 genomes per cell; G, FF366-3, a cell line established from HUCL by transfection with EBV DNA.

† R^2, estimate of fit to a linear model (13).

‡ n, Number of sample spots used to obtain estimates of genome number and to calculate R^2.

§ CN_{50}, cell number per spot at which 50% of the spots would register a positive response.

¶ Picograms of EBV DNA estimated to be contained in a spot registering a positive response above background.

To determine independently the EBV genome content of Raji cells, the reaction was standardized by mixing known amounts of purified EBV DNA with uninfected mononuclear leukocytes. In one such experiment the value obtained for Raji cells was 109 EBV DNA copies per cell, about twice the value usually published. We considered the possibility that the cell count inadequately reflected the cellular DNA content of each spot, because of inaccuracies in cell counting or because the proportion of Raji cells that had passed S phase was ignored.

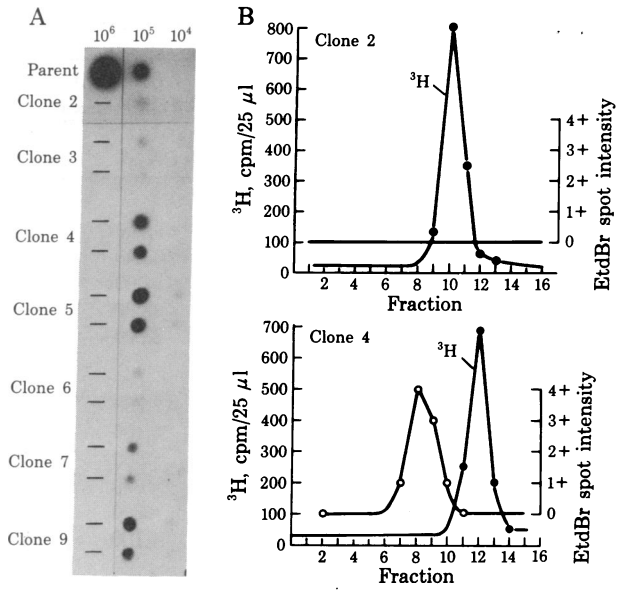

FIG. 5. Selection of clones that are producers of viral DNA. (*A*) Autoradiographic responses of spots of individual clones. All clones were spotted in duplicate except clone 2, for which the duplicate was technically unsatisfactory. (*B*) CsCl gradients of attempted preparation of viral DNA from clones 2 and 4. ^{3}H cpm in *Bacillus subtilis* DNA used as a marker for DNA of cellular density and ethidium bromide spot intensity according to the method of Sugden (25) are plotted.

Therefore the DNA content of duplicate Raji cell spots was determined by the diphenylamine reaction (23), and the EBV genome content per cell was calculated on the basis of the estimate that each cell contains 6 pg of DNA. In this experiment Raji cells were found to contain 98 genome equivalents per cell. This estimate is higher than that generally reported (17).

Purified viral DNA used in the artificial standard may bind to the filter less efficiently or may elute more readily than whole cell DNA (24). Our subline of Raji cells may be one of those with a higher genome content (17).

Sensitivity. In four experiments we determined the smallest number of cells of the different lymphoid lines that could be detected above background hybridization (Table 1). For those lines with low average genome numbers per cell (median 5) between 2200 and 88,400 cells per spot were required; producer lines were always detected at the lowest dilution assayed, 800–7400 cells per spot. The EBV DNA content of the spot containing the least number of cells that would yield a positive hybridization response was calculated. These average values were 49, 5, 39, and 6 pg of EBV DNA.

Application of Spot Hybridization to the Production of Virions and EBV DNA. Because the yield of EBV from even the best producer lines is low, we explored the value of the spot hybridization technique in the identification of those sublines that were the best yielders of EBV DNA. The level of viral capsid antigen in a series of seven clones of the FF41-1 line was about the same (8–10% cells positive). However, there was considerable variation in their content of viral DNA, as ascertained by the spot hybridization method (Fig. 5*A*). The highest concentration of viral DNA was found in clones 4, 5, and 9, and the lowest in clones 2, 3, and 6. About 1–2 μg of EBV DNA could be harvested per liter of supernatant fluids of clones 4 and 5 but DNA could not be prepared from clone 2 (Fig. 5*B*).

Spot hybridization was used to determine the optimal time for harvest of EBV from two producer marmoset cell lines, B95-8 and CC34-5 (Fig. 6), treated with 12-*O*-tetradecanoylphorbol 13-acetate. Cell growth, cell-associated EBV DNA, and

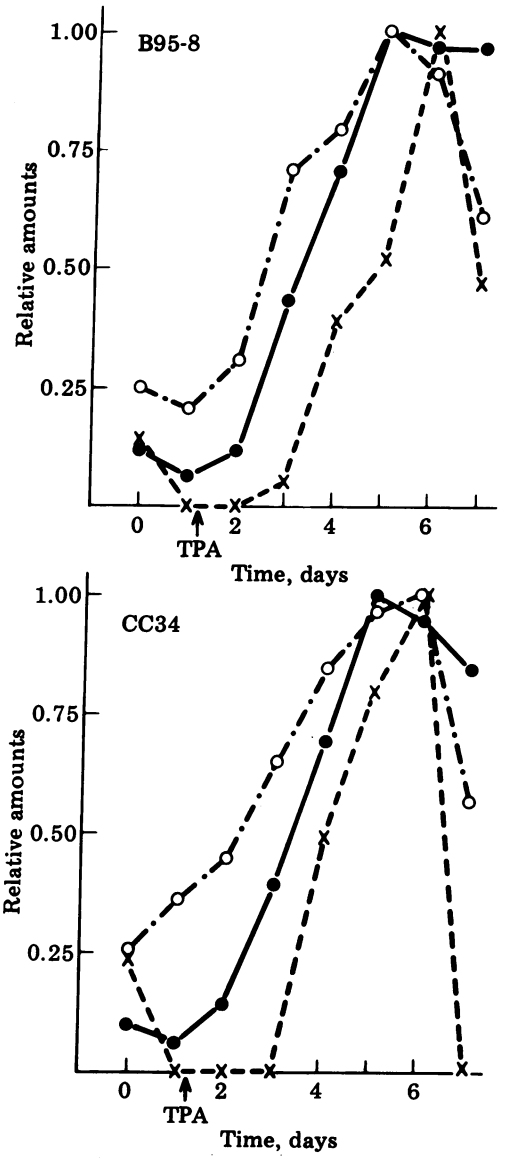

FIG. 6. Kinetics of viral DNA production in two marmoset lines that are producers of EBV. On day 0 cells were adjusted to 4×10^5 per ml in fresh medium. Daily, 1.5 ml of supernatant fluid was clarified of cells by centrifugation (15,600 × *g* for 3 min) and then concentrated to 11 μl by centrifugation (15,600 × *g* for 15 min). Two 5-μl spots were made from each concentrate. Five replicates of 10^5 cells each were spotted daily. On day 1 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was added at 10 nM. Data are expressed relative to the maximal level reached. The maximal cell concentration (○) for both lines was 1.6×10^6 per ml. The maximal cpm bound above background per 10^5 cells (●) was 314 cpm for B95-8 and 207 cpm for CC34-5. The maximal cpm bound above background per 0.7 ml of supernatant fluid (X) was 100 cpm for B95-8 and 23 cpm for CC34-5.

the amount of EBV DNA in the extracellular fluid were monitored daily. There was 4-fold amplification of the amount of cell-associated viral DNA during the exponential phase of cell growth. Viral DNA was not detected in the supernatant until day 4, rapidly reached a maximum on day 6, and fell to low levels on day 7. Thus under these conditions the spot hybridization method clearly identifies the ideal time to harvest virus.

Finally, the genome content of cell-free virus stocks to be used in biologic experiments can be rapidly titrated by spot hybridization. For example, we determined that four different 100-fold concentrated virus stocks prepared from the MCUV line contained between 5 and 32×10^8 genomes per ml. This

value is very close to that determined previously by electron microscopic counts of virions or determination of viral DNA content by reassociation kinetics (20, 25).

DISCUSSION

We have shown that the spot hybridization technique can be used in a quantitative fashion to detect the content of EBV DNA in a cellular sample. The method has several advantages over other hybridization methods, which should make it applicable to epidemiologic studies, diagnostic virology, and a variety of other experiments in which it is desirable to screen multiple samples for viral DNA.

Because no further manipulation of sample DNA is needed, very few cells can be used. The method allows the testing of many samples with a single probe of modest specific radioactivity. Because the hybridizations can so easily be performed, large numbers of replicates can be included and the results can be analyzed accurately with statistical methods.

Spot hybridization is at least as sensitive as the other available methods. In the experiments thus far, the sensitivity of the technique is between 5 and 49 pg of EBV DNA or between 3 and 30×10^4 genomes (Table 1). The relationship between cpm and the number of cells per spot is linear at least up to about 5×10^5 cells per spot (Fig. 4). Therefore the method may detect as few as 0.06 to 0.6 EBV genome equivalents per cell, which approximates the sensitivity of reassociation kinetics (26). The lower limits of detection will be influenced by the number of spotted samples that are analyzed statistically. Increasing the amount of radioactivity in the probes should also increase sensitivity.

The extent of background hybridization with a spot containing EBV-negative cells obviously influences the sensitivity of the technique. Background hybridization has also been seen with the other nucleic acid hybridization techniques, which employ pure DNA; therefore it is unlikely that it is due mainly to binding by cellular constituents other than DNA found on the spot. Nonspecific binding does not occur if the spots are prepared with human erythrocytes. Nonspecific binding by EBV-negative cells is reduced by DNase treatment. These findings suggest that the background may be due to "trapping" of probe DNA by cellular DNA, to a small degree of random homology between EBV DNA and cellular DNA, to some specific homology between certain regions of EBV DNA and cellular DNA, or to contamination of probe DNA with low levels of cellular DNA. Further experiments with probes prepared from specific EBV DNA fragments, propagated by recombinant DNA techniques, may help to clarify the reasons for background binding.

There are obviously certain types of experiments for which spot hybridization is not suitable. The method does not permit measurement of variation in the synthesis of viral DNA from cell to cell, as does *in situ* hybrization. However, in the EBV system the latter technique is usually sensitive only to cells actively producing virus, and it does not detect nonproducer cells, unless they contain large numbers of latent genomes, as do Raji cells (27). Hybridization of Southern blots gives additional information about the size of the viral DNA fragments detected. Such information can be obtained by the spot hybridization method by the use of probes prepared from specific viral DNA fragments.

Because of the simplicity of the method we envision a large number of applications. We have already shown (Figs. 5 and 6) how the technique may be used for screening for cell clones that are high producers of viral DNA and for identifying the time of maximal yield of viral DNA. This is of practical value. The technique should enable more detailed study of virus–cell relationships, for example in timing the onset of EBV genome amplification in relation to transformation. It may be useful in screening for viral mutants that are less restricted in replication of their DNA.

The method should enable further study of the epidemiology and pathogenesis of EBV infections. It may be possible to examine presumed sites of viral excretion and persistence for their genome content. The method can be adapted for analysis of blood leukocytes, bone marrow, and lymphoid and other biopsy tissue so that specimens from individuals with a wide range of diseases can be examined for their EBV DNA content.

The method is not limited to EBV. Any cell population or fluid can be screened by spot hybridization for the presence of a DNA sequence for which a radioisotopically labeled specific probe is available.

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