Suitability of Epstein-Barr Virus DNA Obtained from Superinfected Raji Cells for Complementary RNA Hybridization Studies

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SUMMARY

A method was established to obtain a high yield of Epstein-Barr virus (EBV) DNA for nucleic acid hybridization studies on latent virus DNA in transformed cells. Superinfection of Raji cells with EBV concentrated from HRI cell cultures produced a 600-fold higher yield of EBV DNA than direct isolation of EBV from HRI cell cultures. The virus DNA thus prepared from Raji cells superinfected with EBV was radioisotopically and spectrophotometrically pure and served as a satisfactory template for the preparation of cRNA specific to EBV DNA.

Nucleic acid hybridization is a powerful tool in studying the latency of Epstein-Barr virus genomes. Complementary RNA hybridization (cRNA hybridization) and DNA-DNA reassociation kinetics have been well established in the last five years (Nonoyama & Pagano, 1971; zur Hausen et al., 1972; Nonoyama & Pagano, 1973). The difficulty in carrying out EBV DNA hybridization studies, however, is caused by poor yields of virus particles from productive HRI cells which have been the sole source of EBV DNA. $10^{10}$ HRI cells (virus productive cells) only yielded 1 to 5 µg of virus DNA. Recently an efficient system of EBV superinfection has been established in which virus DNA replication is enhanced and cellular DNA synthesis inhibited (Yajima & Nonoyama, 1976). The virus DNA directly extracted from the superinfected cells is pure enough to prepare cRNA specific to EBV DNA. The method is simple, inexpensive and recommended for routine studies of detection of EBV DNA in tumour tissues and cells in any laboratory.

HRI cells, virus productive cells (derived from a Burkitt's lymphoma patient), Raji cells, virus non-productive cells (also derived from a Burkitt's lymphoma patient) which contained 50 EBV genomes/cell and Simpson cells, EBV genome-negative human myeloblastic cells, were cultivated in RPMI 1640 with 5% calf serum (CS). HRI cells were grown to $10^6$/ml in 100 ml of RPMI 1640 at 35 °C and cells were removed by centrifuging at 8000 rev/min for 10 min. Virus was pelleted from the supernatant fluid at 16000 rev/min for 1 h at 4 °C in a Beckman J21 centrifuge and suspended in 1 ml of phosphate-free minimum essential medium (P-MEM). Raji cells ($10^6$/ml) in 10 ml of RPMI 1640 with 5% CS were harvested and suspended in 1 ml of the virus suspension. After adsorption of the virus to the cells for 1 h at room temperature, 9 ml of pre-warmed P-MEM with 2% CS were added to the cells and the suspension incubated at 37 °C. Multiplicity of infection per cell was 2 to 3 as determined by an early antigen forming unit assay (EA FU: Nonoyama & Pagano, 1972). $^3$H-thymidine (1 µCi/ml) was added to the culture 12 h post infection (p.i.) and the superinfected cells were harvested at 40 h p.i. at 5000 rev/min for 10 min, resuspended in 2 ml of TNE (tris pH 9, 0.01 M, EDTA, 0.001 M, NaCl, 0.15 M) and pronase (1 mg/ml) and sarkosyl (1%) were added. After incubation at 37 °C for 6 h, the DNA was extracted by shaking with water-saturated phenol and precipitated from the aqueous phase by addition of 2 vol. of ice-cold ethanol. The DNA was suspended in 0.0025 M-EDTA and CsCl added.
Superinfection of Raji cells with EBV in P-MEM caused complete inhibition of cellular DNA synthesis and all the DNA synthesized was found to be virus DNA (Fig. 1). As yields were not consistently high when superinfected cells were maintained in RPMI 1640 medium, all subsequent experiments were done in P-MEM (Yajima & Nonoyama, 1976). Recovery of cellular DNA was approx. one tenth of the amount obtained from mock infected cells to a final density of 1.70 g/ml. Centrifugation was carried out at 38000 rev/min for 60 h at 18 °C in a type 50 rotor in a Beckman L3-50 centrifuge and 2 drop fractions collected by puncturing the bottom of the tube. Radioactivity was counted in a Packard Scintillation Counter and extinction was measured at 260 nm in a Beckman model 24 spectrophotometer. The virus DNA peak fractions were pooled and recentrifuged in CsCl as above. 3H-EBV DNA and non-radioactive EBV DNA from purified virions obtained from larger scale cultures of HRI cells were prepared as described by Nonoyama & Pagano (1971). The method of cRNA preparation and the condition of cRNA hybridization have been described (Nonoyama & Pagano, 1971). Pre-adsorption of cRNA has been carried out as follows. Approx. 200 μg of Simpson DNA was fixed to a nitrocellulose filter and four stacks of filters were soaked with 0.3 to 0.4 ml of cRNA solution containing 6× SSC and 0.1% sarkosyl. The mixture was incubated for 6 h at 66 °C and the filters were washed with 6× SSC. Approx. 70% of the radioactivity was recovered after the pre-adsorption.

Fig. 1. CsCl equilibrium centrifugation of DNA extracted from Raji cells superinfected with EBV. Raji cells were superinfected with EBV and DNA was extracted and centrifuged in CsCl at 38000 rev/min for 60 h at 18 °C in a type 50 rotor in a Beckman L3-50 centrifuge. After fractionation into 80 tubes, the refractive index was measured for each fraction and 0.5 ml of TNE was added to measure extinction at 260 nm. A portion of each fraction was counted for radioactivity in a Packard tricarb scintillation counter. ●, Virus DNA; ○, cell DNA.
Fig. 2. Hybridization efficiency of cRNA with Raji cell and B95 cell DNA. Various amounts of Raji, B95, and Simpson cell DNA were fixed to nitrocellulose filters and hybridized with $1.5 \times 10^5$ cts/min of cRNA as described in the text. (a) cRNA prepared from virus DNA isolated from superinfected Raji cells. (b) cRNA prepared from virus DNA isolated from purified virions. ○——○, Raji cell DNA; △—△, B95 cell DNA; ●——●, Simpson cell DNA; ◦—◦, Simpson cell DNA; cRNA used without pre-adsorption.

indicating that superinfection of Raji cells with EBV also caused degradation of cellular DNA which made isolation of virus DNA easy. The peak fractions were pooled and re-centrifuged in CsCl and a symmetrical radioactive peak was obtained. The virus DNA thus obtained showed, in a Model E analytical centrifuge, a symmetrical peak at the density of 1.718 g/ml calculated from Clostridium (1.681 g/ml) and Micrococcus DNA (1.731 g/ml). The density of the virus DNA obtained was the same as previously reported for purified EBV DNA (Schulte-Holthausen & zur Hausen, 1970). The virus DNA obtained from the superinfection was thus radioactively and spectrophotometrically pure and showed the correct buoyant density of EBV DNA in CsCl centrifugation. The yield of DNA was extraordinarily high and 3 to 5 μg of virus DNA was prepared from $10^7$ infected Raji cells. When compared with direct extraction of virus DNA from virions purified from HRI cultural fluid, the yield of virus DNA per HRI cell from the same HRI culture was amplified 600-fold through superinfection of Raji cells.

Although cRNA hybridization is a useful tool for the study of latency of EBV DNA, it has been difficult to supply enough cRNA for the study because of poor yield of EBV DNA from HRI cultures. Therefore, suitability of this virus DNA for preparation of cRNA specific to EBV DNA was tested. cRNA was prepared both from virus DNA obtained from superinfected Raji cells and from virions purified from large scale cultures of HRI cells. The final incorporation efficiency of $^3$H-UTP was 3.5% of the substrate used. Background
hybridizations of the prepared cRNA to cellular DNA (Simpson DNA) free of virus DNA were about 700 ct/min/50 µg of cellular DNA without pre-adsorption (Fig. 2). After the pre-adsorption of the preparation to stacked filters containing Simpson DNA background hybridization of cRNA prepared from virus DNA isolated from superinfected Raji cells was reduced to 250 ct/min/50 µg cell DNA whereas that from virus DNA isolated from purified virions was reduced to 180 ct/min/50 µg cell DNA. DNA from Raji cells which contained 50 genomes/cell and from B95 cells which became non-productive during cultivation and contained 30 genomes/cell were tested for the efficiency of hybridization with both types of cRNA. As shown in Fig. 2, cRNA prepared from virus DNA isolated from superinfected Raji cells and from purified virions obtained from large scale cultures of HRI cells, hybridized to the same extent to Raji or B95 cell DNA; cRNA prepared from virus DNA isolated from superinfected Raji cells hybridized 2900 ct/min/50 µg HRI DNA and 1660 ct/min/50 µg of B95 cell DNA whereas cRNA prepared from virus DNA obtained from purified virions hybridized 2750 ct/min/50 µg of HRI cell DNA and 1700 ct/min/50 µg of B95 cell DNA. The results show that virus DNA purified from Raji cells superinfected with EBV concentrated from the supernatant of HRI cell cultures can serve as a satisfactory template for making cRNA specific to EBV DNA, when compared with DNA extracted from virus particles. Thus, the superinfection method is simple, quick, economical and can be carried out in any laboratory which is not equipped with instruments such as a continuous flow ultracentrifuge or a continuous vacuum dialysis concentrator to handle large amounts of culture fluid for virus purification. As the yield of virus DNA from HRI cells is amplified approx. 600-fold through superinfection of Raji cells, it requires only 100 ml of HRI culture (10^8 cells) and 10 ml of Raji culture (10^7 cells) to obtain a few µg of virus DNA instead of culturing 50 l of HRI cells. A cycle of the infection can be finished in 2 days (Nonoyama & Pagano, 1972) and the experiment can be repeated immediately in case of a failure of isolation of virus DNA. Considering the results in Fig. 2 that the extent of cRNA hybridization to purified EBV DNA, Raji or B95 cell DNA is not distinguishable between cRNA prepared by virus DNA from superinfected Raji cells and from purified virions, the same type of cRNA specific to virus DNA must have been made. Recent experiments indicate that virus DNA from superinfection retains sequence homology indistinguishable from virus DNA of parental EBV from HRI cells (Yajima & Nonoyama, 1976). This justifies the use of virus DNA from superinfected Raji cells even for DNA-DNA reassociation kinetics study (Tanaka et al. 1976) as well as for a cRNA hybridization study. The direct labelling of virus DNA with ^32P in this system is easy and virus DNA with specific activity of more than 10^6 ct/min/µg can be obtained, which is adequate for DNA-DNA reassociation studies.

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