Analysis and Isolation of Cytomegalovirus DNA by Field Inversion Gel Electrophoresis

By FRANK M. VAN DEN BERG, MEHDI JIWA, REN ROOK AND JAN L. M. C. GEELEN

1 Department of Pathology and 2 Department of Virology, Academic Medical Centre, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

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SUMMARY

High molecular weight human cytomegalovirus (CMV) DNA was isolated from agarose-embedded infected human diploid cells by employing field inversion gel electrophoresis. A high yield of CMV DNA molecules was obtained within 1 week of infecting the cell culture. Labelling of the viral DNA with biotin by nick translation enabled the detection of CMV-infected cells in sections of paraffin-embedded human adrenal gland by in situ hybridization.

Human cytomegalovirus (CMV) has a linear dsDNA genome of 235 kb which is composed of 58% GC base pairs (Geelen et al., 1978; Huang et al., 1973; Stinski et al., 1979). Isolation of total genomic CMV DNA from purified virus particles is a time-consuming and laborious procedure (Geelen et al., 1978) which gives a relatively low yield. In view of the growing interest in virus diagnostics using in situ hybridization (ISH) with non-radioactive probes (Loning et al., 1986; Raap et al., 1987), it is important in the case of suspected CMV infection to be able to use the total virus genome as an ISH probe instead of molecularly cloned DNA fragments, because detection limits appear to be proportional to the total amount of target sequences that are available to be hybridized in situ.

Recently a novel technique has been developed that facilitates separation of DNA molecules in the range of 20 to 2000 kb in a standard electrophoresis chamber (Carle et al., 1986). This field inversion gel electrophoresis (FIGE) technique is based on a computer-operated system in which the high voltage over the gel is applied intermittently backward and forward (pulsed) in a 1 to 3 ratio over an 18 to 24 h period in which the pulse intervals are gradually increased. The different pulse lengths result in a net forward movement of the DNA molecules.

To test whether preparative FIGE could be employed in the isolation of CMV genomes we infected three roller bottles of confluent human diploid embryonic lung cells (50 × 10⁶ cells/bottle) with 0.2 (one bottle) and 0.6 p.f.u./cell (two bottles) of CMV strain AD169 and cultured the cells for 4 or 5 days as previously described (Geelen et al., 1978). Infected cells were collected by trypsinization and centrifugation at 800 g. The cell pellet was immediately resuspended in a small volume of TE buffer (10 mM-Tris-HCl pH 7.5, 10 mM-EDTA) to make a slurry which was warmed to 37 °C and subsequently mixed with an equal volume of fluid agarose (2%) in TE buffer at 37 °C. The cell−agarose mixture (2 to 3 ml) was carefully pipetted onto a clean horizontal microscope slide and left to solidify at room temperature. Subsequently 0.4 × 2 cm slices (called inserts) were cut from the gel and incubated in SE buffer (0.5 M-EDTA, 1% sodium lauryl sarcosinate) for 1 h at room temperature. The buffer was aspirated off and replaced with SE buffer containing 0.5 mg/ml proteinase K (Boehringer). The inserts were then incubated overnight at 37 °C under continuous agitation. The next day the inserts were rinsed in three changes of SE buffer for 6 h and stored in SE buffer at 4 °C.

To test for the presence of CMV DNA in the inserts, small (0.4 × 0.7 cm) pieces of insert were loaded into a 14-slot FIGE gel in parallel with similarly prepared inserts of herpes simplex virus...
Fig. 1. FIGE analysis of CMV (human strain AD169)-infected cell DNA prepared in agarose blocks (inserts). Inserts containing different DNA samples prepared as described in the text were loaded into the holes of a 1.5% agarose slab gel with the aid of a spatula. Electrophoresis was carried out for 25 h at 8 V/cm and 14°C using linearly increasing pulse times from 5 to 50 s in the forward direction. After EtBr staining and u.v. photography (a) the DNA pattern was transferred to a nitrocellulose membrane, hybridized with the 32P-labelled EcoRI J fragment of CMV (Boom et al., 1986), washed at high stringency (20 mM-Na+, 65°C) and autoradiographed (b). Lanes 1, S. cerevisiae (strain 303) chromosomes; lanes 2, uninfected human fibroblasts; lanes 3, HSV-2-infected cells; lanes 4 to 6, CMV-infected cells (0.6 p.f.u./cell 5 days, 0.6 p.f.u./cell 4 days and 0.2 p.f.u./cell 4 days respectively); lanes 7, phage lambda ladders (Carle & Olson, 1984); lane 8, T. brucei chromosomes.
the full length CMV genome. The relative amount of intracellular CMV DNA had apparently increased between 4 and 5 days post-infection although the differences between lanes 4, 5 (and 6) may have resulted in part from variations in the cell number and microclimate in the three roller bottles.

The genome sizes calculated from Fig. 1 (a) for both HSV and CMV using the leading front of each band as the measuring point are slightly lower than the generally accepted values of 155 and 235 kb. This discrepancy could be due to the relatively high GC base pair content of these herpesvirus DNAs which results in a more compact and therefore shorter double helical configuration (Freifelder, 1970).

To demonstrate that the u.v. fluorescent bands in lanes 4 and 5 (Fig. 1 a) represented the CMV genome, the gel was Southern-blotted onto nitrocellulose using standard procedures (Van den Berg et al., 1985) and hybridized with the $^{32}$P-labelled cloned EcoRI J fragment of CMV (Boom et al., 1986). Comparison of the u.v. photograph (Fig. 1 a) and the autoradiogram (Fig. 1 b) allowed several conclusions. (i) Four days after infection at 0-2 p.f.u./cell, genome length CMV DNA is not yet detectable. (ii) The DNA smear in (Fig. 1 a) lanes 4, 5 and 6 is predominantly of cellular origin possibly resulting from CMV-induced degradation of the host cell genome (Lileci et al., 1980). (iii) The calculated sizes for the CMV-specific bands (from bottom to top 225, 265, 465, 500 and 690 kb) strongly suggest that during productive infection the CMV genome exists in multimeric forms; this could imply that the CMV genome replicates via a rolling circle mechanism as has been suggested for HSV (Roizman, 1979). In addition there appear to be aberrantly sized (defective) CMV genomes (265 kb) that also occur in a dimeric form (500 kb). (iv) Replication of CMV and HSV may not proceed according to entirely identical mechanisms (Spaete & Mocarski, 1985) since in the case of HSV we have been unable to detect multimeric forms at different stages of the infectious cycle in u.v. photographs of FIGE gels (see Fig. 1 a, lane 3) or upon blot hybridization of these gels (unpublished observations). Due to the faster replication cycle of HSV compared with CMV this result may stem from the quicker processing of replicative intermediates of HSV.

To isolate the CMV DNA from the infected cell inserts, preparative FIGE was performed essentially as in Fig. 1 except that a 12 cm wide slot was used which could hold about one-third of the inserts prepared from the 0-6 p.f.u./cell bottles (about $15 \times 10^6$ infected cells). After brief staining with EtBr the CMV DNA band was excised with a scalpel under low intensity u.v. illumination. The gel slice was placed in dialysis tubing containing 0-5 x TBE buffer and the CMV DNA was electro-eluted and purified as described by Maniatis et al. (1982) except that FIGE electro-elution was employed. The yield was very high; up to 100 $\mu$g CMV DNA was obtained from one roller bottle ($50 \times 10^6$ cells), which is more than 10 times the amount routinely prepared after virion isolation (Geelen et al., 1978). When the CMV DNA preparation was analysed by a second cycle of FIGE some breakdown was observed, but the CMV DNA still ranged in size from 50 to 150 kb (not shown). Breakdown of the CMV genomes probably occurred during the post-electrophoresis purification and concentration steps where no special care was taken to conserve intact genomes.

It could be expected from the results described in Fig. 1 that the CMV DNA preparation obtained by the FIGE protocol described here contained contaminating human DNA sequences from the background smear. To analyse the extent of contamination we performed filter hybridization using FIGE-purified CMV DNA and CMV virion DNA (Geelen et al., 1978) as probes (Fig. 2). Stepwise dilutions of denatured human DNA (1500 to 0.05 ng; lanes 1) and CMV virion DNA (3000 to 1 pg; lanes 2) were spotted in parallel onto duplicate nitrocellulose filter strips (a and b). Filter a was hybridized with $^{32}$P-labelled FIGE-purified CMV DNA and filter b was hybridized with $^{32}$P-labelled CMV virion DNA. The results in Fig. 2 demonstrate that the FIGE-purified CMV DNA detects 0-5 ng human DNA and 3 pg CMV DNA after overnight exposure of the autoradiogram (filter a), while virion DNA detects 5 ng human DNA and 1 pg CMV DNA (filter b). The latter results from the known cross-reactivity of parts of herpesvirus genomes with eukaryotic DNAs. Having corrected for the difference in detection levels of CMV DNA with each probe in Fig. 2 we estimated that the FIGE-purified CMV DNA preparation effectively contains about 3% human DNA contamination and the
Fig. 2. Filter hybridization analysis of the purity of CMV DNA isolated by the FIGE procedure. Sequential dilutions in 1 M-ammonium acetate of the indicated amounts of denatured human DNA (lanes 1) and of CMV DNA isolated from purified virions (lanes 2) were spotted in duplicate onto nitrocellulose filter strips which were presoaked in 20 × SSC and air-dried. Filters a and b were hybridized with 0.2 μg FIGE CMV DNA and 0.2 μg CMV virion DNA labelled with 32P to a specific activity of 10⁸ c.p.m./μg respectively. Then Fuji RX film was exposed for 20 h at −96 °C using Dupont Cronex Lightning-plus intensifying screens.

hybridization detection sensitivity for CMV DNA is about 150 times better than for human DNA.

To test whether FIGE-purified CMV DNA could be employed as a probe in diagnostic ISH the DNA was labelled with biotin by nick translation using biotin-16-dUTP (Enzo Biochemicals, New York, N.Y., U.S.A.). ISH was performed on sections of a paraffin-embedded adrenal gland from an acquired immune deficiency syndrome (AIDS) patient who died showing signs of CMV infection. Paraffin was removed from sections mounted on gelatin-coated microscope slides; this was followed by sequential incubation in 0-2 M-HCl for 10 min at room temperature and in 50 μg/ml proteinase K (Boehringer) for 30 min at 37 °C. After dehydration and drying, sections were prehybridized in HM [2 × SSC (SSC is 0-15 M-NaCl, 0-05 M-trisodium citrate, pH 7-0), 50 % formamide, 250 μg/ml denatured salmon sperm DNA, 10 × Denhardt’s solution, 10 % dextran sulphate] for 1 h at 37 °C and subsequently rinsed in 2 × SSC, dehydrated and air-dried. Biotin-labelled FIGE-purified CMV DNA or an HSV type 2 DNA probe (6 ng) in 10 μl HM was applied to the sections which were subsequently covered with a glass coverslip. Probe and target DNAs were denatured by placing the slide for 10 min on an 80 °C hotplate. Hybridization was then performed for 18 h in a sealed humidified chamber at 37 °C. Coverslips were removed and sections were thoroughly rinsed in 0-5 × SSC at room temperature after which positive hybridization was visualized by staining the biotin-labelled DNA in the sections with a commercially available streptavidin/poly-alkaline-phosphatase kit (Bethesda Research Laboratories) using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as the substrate. In haematoxylin- and eosin-stained sections of both the cortex and medulla of the adrenal gland characteristic cells were found with large rounded or oval inclusion bodies which indicated CMV infection (Fig. 3a). After ISH with the biotin-labelled FIGE-purified CMV DNA these cells were intensely stained (Fig. 3b) contrasting with the lack of staining when using a biotin-labelled HSV-2 DNA probe (not shown). In addition, cells are seen in Fig. 3(b) that stain positively for CMV DNA but are morphologically normal, which demonstrates the sensitivity and diagnostic power of ISH using the total CMV genome as probe. The human DNA contaminating the FIGE-purified CMV DNA preparation apparently
Fig. 3. In situ hybridization using biotin-labelled FIGE-purified CMV DNA on paraffin-embedded CMV-infected adrenal gland. CMV DNA was biotin-labelled by nick translation with biotin-16-dUTP and hybridized to 6 μm sections of paraffin-embedded adrenal gland tissue from an AIDS patient as described in the text. (a) Haematoxylin and eosin stain; (b) ISH with biotin-labelled FIGE CMV probe detected with NBT/BCIP. Bar marker represents 50 μm.

did not interfere with the ISH procedure (Fig. 3a), as the nuclei of uninfected cells remained unstained. A highly purified CMV DNA preparation can be obtained by directly dissolving the agarose slice containing the viral DNA from the FIGE gel in sodium iodide and subsequently separating the CMV DNA from human DNA sequences by equilibrium density centrifugation in the presence of EtBr.

At present we cannot measure the sensitivity of the ISH procedure because estimation of the CMV genome copy number in the infected cells in a paraffin-embedded tissue sample is not possible. However, we have observed morphologically normal cells with a single spot of positive hybridization signal indicating a relatively early stage of infection.

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