Cloning and sequencing of a highly productive, endotheliotropic virus strain derived from human cytomegalovirus TB40/E

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Human cytomegalovirus (HCMV) strain TB40/E, replicates efficiently, exhibits a broad cell tropism and is widely used for infection of endothelial cells and monocyte-derived cells yet has not been available in a phenotypically homogeneous form compatible with genetic analysis. To overcome this problem, we cloned the TB40/E strain into a bacterial artificial chromosome (BAC) vector. Both highly endotheliotropic and poorly endotheliotropic virus clones, representing three distinct restriction fragment patterns, were reconstituted after transfection of BAC clones derived from previously plaque-purified strain TB40/E. For one of the highly endotheliotropic clones, TB40-BAC4, we provide the genome sequence. Two BACs with identical restriction fragment patterns but different cell tropism were further analysed in the UL128-UL131A gene region. Sequence analysis revealed one coding-relevant adenine insertion at position 332 of UL128 in the BAC of the poorly endotheliotropic virus, which caused a frameshift in the C-terminal part of the coding sequence. Removal of this insertion by markerless mutagenesis restored the highly endotheliotropic phenotype, indicating that the loss of endothelial cell tropism was caused by this insertion. In conclusion, HCMV strain TB40/E, which combines the high endothelial cell tropism of a clinical isolate with the high titre growth of a cell culture adapted strain, is now available as a BAC clone suitable for genetic engineering. The results also suggest BAC cloning as a suitable method for selection of genetically defined virus clones.

INTRODUCTION

One of the hallmarks of human cytomegalovirus (HCMV) is its very broad cell tropism in vivo, which allows for systemic spread of this herpesvirus into virtually any organ during acute infection. Therefore, there has been increasing interest in studying HCMV infection of endothelial cells (EC) and monocyte-derived cells, which are assumed to contribute greatly to the haematogenous spread of the virus (Gerna et al., 2002; Hertel et al., 2003; Jarvis & Nelson, 2002; Sinzger & Jahn, 1996). This has been facilitated by the introduction of EC-propagated HCMV strains that have preserved their natural broad cell tropism in cell culture. One of these strains, TB40/E, has been used by several laboratories as a highly endotheliotropic and macrophage-tropic strain (Allal et al., 2004; Bentz et al., 2006; Hertel et al., 2003; Homman-Loudiyi et al., 2003; King et al., 2006; Moutafsis et al., 2004; Reeves et al., 2005; Reinhardt et al., 2005). However, genetic analyses have revealed that this strain is genetically heterogeneous despite several rounds of plaque purification, which argued against its use for genotype–phenotype analyses (Dolan et al., 2004). Generation of enhanced green fluorescent protein-tagged mutants of TB40/E by conventional homologous recombination resulted in random selection of variants with either high or low EC tropism (Laib Sampaio et al., 2005), indicating that it is not only genotypically but also phenotypically heterogeneous.

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The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is EF999921.
Genetic investigation of cytomegaloviruses (CMV) has improved because of cloning of CMV genomes into bacterial artificial chromosomes (BAC) (Borst et al., 1999; Messerle et al., 1997). This technique (i) greatly facilitates genetic manipulation of viral genes in the context of the viral genome, (ii) enables amplification of the genome in the absence of selective pressure, and (iii) yields clonal viral genomes without the need of plaque purifications (Brunner et al., 1999; Messerle et al., 2000). Here, we report generation of BACs derived from TB40/E in order to yield genetically pure and highly endotheliotropic clones of this virus and to test the hypothesis that the phenotype of variants contained within TB40/E is determined by the UL128-UL131A gene region previously linked to loss of EC tropism during extended fibroblast adaptation (Adler et al., 2006; Hahn et al., 2004; Wang & Shenk, 2005b).

**METHODS**

**Cells.** Human foreskin fibroblasts (HFF) were cultured in minimum essential medium (MEM; Gibco) containing 5% fetal calf serum (FCS), 2.4 mmol glutamine l⁻¹ and 100 μg gentamicin ml⁻¹ (designated fibroblast medium), and were used for experiments at passage 10–25. Human umbilical vein endothelial cells (HUVEC) were cultured in RPMI 1640 containing gentamicin (100 μg ml⁻¹), heparin (5 IU ml⁻¹), endothelial cell growth supplement (50 μg ml⁻¹; Becton Dickinson) and human serum (10%; seronegative for HCMV) (designated EC medium), and were used for experiments at passage 4–7. All cell preparations tested negative by 4,6-diamidino-2-phenylindole (DAPI) staining for mycoplasmas.

**Viruses.** HCMV strain TB40/E was derived in our laboratory from throat wash of a bone marrow transplant recipient by propagation for 5 passages in fibroblasts and 22 passages in EC (Sinzger et al., 1999). For preparation of virus stocks, HFF were infected at an m.o.i. of 0.1 p.f.u. per cell. Supernatants of infected cultures were harvested 6 days after infection and stored at −80 °C after removal of cell debris by centrifugation for 10 min at 2800 g. The infectious titre in HCMV preparations was determined by TCID₅₀ assays in fibroblasts on 96-well plates (Mahy & Kangro, 1996).

**Generation of BACs.** The EC-propagated HCMV strain TB40/E was cloned as a BAC in Escherichia coli as described previously (Hahn et al., 2002). Briefly, 10⁶ HFF were transfected with 35 μg plasmid pEB1097 containing a tk-gpt-bac-cassette flanked with HCMV homologous sequences of US1-US2 (AD169 nt 192648–193360; GenBank accession no. X17403) on the right side and US6-US7 (AD169 nt 195705–197398) on the left side of the cassette. After 24 h, the monolayer was washed to TB40/E at an m.o.i. of 5. Three rounds of selection with 100 μM xanthine and 25 μM mycophenolic acid followed. Circular episomal DNA was extracted using the method of Hirt (1967) and electroporated into E. coli as described previously (Hahn et al., 1999; Messerle et al., 1997). The infectious titre in HCMV preparations was determined by TCID₅₀ assays in fibroblasts on 96-well plates (Mahy & Kangro, 1996).

**RFLA.** Viral DNA was digested with either EcoRI, BamHI, Xhol or HindIII. DNA samples were then separated by electrophoresis on 1% agarose gels, stained with ethidium bromide and visualized by transillumination with UV light.

**Focus expansion (FE) assays.** The capability of reconstituted viruses to grow in EC cultures was quantified by FE assays as described previously (Sinzger et al., 1997). Briefly, frozen infected cells were thawed, washed and co-cultured in 96-well plates together with either uninfected HFF or uninfected HUVEC. To quantify HCMV replication in EC cultures (FEHUVEC), 2×10⁴ uninfected HUVEC per well were co-cultured with serial dilutions of infected fibroblasts (10⁻²–10⁻⁵) for 5 days at 37 °C with 5% CO₂ in EC medium. To determine HCMV replication in fibroblast cultures (FEHFF), 2×10⁴ uninfected HFF per well were co-cultured in the same way, using fibroblast medium. After 5 days of co-cultivation, cells were fixed with cold methanol and HCMV immediate-early (IE) antigen was detected by indirect immunoperoxidase staining with monoclonal antibody E13 (Biosoft), peroxidase-conjugated goat anti-mouse-IgG Fab’, and the chromogen diaminobenzidine (Sigma). All tests were done in quadruplicate. Stained dishes were analysed with an Axiovert 135 microscope (Zeiss). Infectious foci were defined as clusters of three or more antigen-positive cells. The number of infected cells in the largest focus in each of the four parallel tests was counted. The highest and the lowest counts were always eliminated and the mean values of the remaining two counts were defined as the FE value of the respective strain, thus ensuring highly reliable results (Sinzger et al., 1997).

**Determination of infection efficiency with cell-free virus.** HFF and HUVEC grown on gelatin-coated 96-well plates (μclear; Greiner) were washed with fresh MEM medium with 5% FCS for 30 min and then incubated with the respective virus preparation at an m.o.i. of 0.7. After infection (2 h), virus preparations were replaced by fresh cell culture medium and cells were incubated overnight. Cells were...
then fixed with 80% acetone for 5 min at room temperature. For immunofluorescence detection of infected cells, the fixed cultures were subsequently incubated with monoclonal antibody (mAb) E13, directed against HCMV-IE antigen (Biosoft) and Cy3-conjugated goat anti-mouse-IgG Fab’2 (Jackson ImmunoResearch). Finally, nuclei were counterstained with DAPI. Stainings were read under a Zeiss Axiovert 200 microscope and documented using Axiovision software.

Nuclear localization assay. HFF and HUVEC grown on gelatin-coated 96-well plates (µclear; Greiner) were washed with fresh MEM medium with 5% FCS for 30 min and then incubated with the respective virus preparation at an m.o.i. of 5 p.f.u. per cell. After infection (1 h), virus preparations were replaced by fresh cell culture medium and cells were incubated for an additional 5 h. Cells were then fixed with 80% acetone for 5 min at room temperature. For immunofluorescence detection of virus particles, the fixed cultures were subsequently incubated with mAb XP1 directed against the capsid-associated HCMV- tegument protein pp130 (Behringwerke) and Alexa Fluor 488-conjugated goat anti-mouse-IgG Fab’2 (Molecular Probes). Remaining binding sites for mouse antibodies were blocked with mouse serum. For immunofluorescence detection of microtubules, cells were then incubated with Cy3-labelled mouse anti-β-tubulin (Sigma). Finally, nuclei were counterstained with DAPI. Stainings were visualized under a Zeiss Axiovert 200 microscope and documented using Axiovision software.

DNA sequence analyses. Sequence comparison of the UL128-UL131A gene region from TB40-BAC1 and TB40-BAC4 was done by SequiServe. Complete sequencing of TB40-BAC4 was done by Macrogen custom sequencing service using shotgun sequencing with six times coverage and primer walking to fill the remaining gaps. Sequence data were analysed and aligned using CLUSTAL W (Chenna et al., 2003) and BioEdit v.5.0.9 (Hall, 2001). The nucleotide sequence was annotated using Lasergene SeqBuilder software (DNASTAR) and deposited at GenBank (accession no. EF999921).

RESULTS

Generation, reconstitution and phenotypic characterization of BAC clones from HCMV strain TB40/E

BAC clones were generated from the highly endotheliotropic HCMV strain TB40/E in order to serve two purposes: (i) the availability of TB40/E-derived BACs would greatly facilitate genetic manipulations of this virus; (ii) reconstitution of virus from TB40/E-derived BAC clones would overcome the previously reported problem of genetic heterogeneity found within TB40/E despite repeated rounds of plaque purifications.

For the generation of TB40/E-derived BACs, the US2-US6 genome region of the viral genome was replaced with the selectable F-ori-containing plasmid pEB1997 by homologous recombination in TB40/E-infected HFFs, and successfully recombined viral genomes were enriched by selection with xanthine/mycophenolic acid. Circular viral DNA was extracted from infected HFFs and transformed into E. coli DH10B. After transfection of DNA from 18 bacterial clones into HFFs, infectious virus could be reconstituted from all TB40-BACs, and based on an initial RFLA nine TB40-BACs were chosen for further phenotypic and genotypic analyses. RFLAs of DNA from these viruses confirmed the previously reported finding that TB40/E was not genetically homogeneous despite repeated plaque purifications. Three distinct restriction fragment profiles were found after digestion with enzymes BamHI, EcoRI, HindIII and XbaI (Table 1). Profile 1 was represented by three HCMV-TB40-BAC clones (1, 2 and 4); profile 2 was represented by five HCMV-TB40-BAC clones (3, 6, 10, 12 and 18); and profile 3 was only represented by HCMV-TB40-BAC clone 9. Differences are not due to different orientations of US and UL segments in the TB40-BAC clones, as RFLAs have been performed with the reconstituted viruses that are known to contain all four isoforms at equal stoichiometry (Kilpatrick & Huang, 1977, McVoy & Ramnarain, 2000). Therefore, the three RFLA profiles most likely represent true genetic variants present in TB40/E.

The EC tropism of all successfully reconstituted infectious clones was determined by FE assays. Only two virus clones (reconstituted from TB40-BAC4 and TB40-BAC12) could spread efficiently in HUVEC monolayers as determined by the number of infected cells per focus after 7 days of cultivation. (Fig. 1a). Unexpectedly, the genetic pattern as determined by RFLA did not correlate with the phenotype

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Table 1. Genotype and phenotype of viruses reconstituted from TB40/E-derived BACs

RFLA profile: for each enzyme, the different restriction fragment patterns are assigned with capital letters in the order of appearance. The three different combinations of restriction profiles were assigned with numbers in the order of appearance. EC tropism: the number of infected cells/focus after 7 days of focal expansion from one productively infected cell in HUVEC monolayers is given as FEHUVEC.
(Table 1). For further phenotypic characterization, reconstituted viruses HCMV-TB40-BAC1 and HCMV-TB40-BAC4 were chosen, because they represented a maximal difference in EC tropism on the background of an identical RLFA pattern (Fig. 1b), thus making them particularly suitable for genotypic comparisons.

To test whether HCMV-TB40-BAC4 and HCMV-TB40-BAC1 were suitable to represent the phenotypic differences described previously for the parental strains TB40/E and its poorly endotheliotropic counterpart TB40/F, respectively (Sinzger et al., 2000), we analysed the infection capacity of cell-free virus in HUVEC cultures and the efficiency of nuclear translocation. As expected, the phenotypic difference in FE assays was also reflected in the 'cell-free' infection mode. When HUVEC were infected for 24 h at an m.o.i. of 0.7 p.f.u. per cell as normalized on HFF, only HCMV-TB40-BAC4 infected HUVEC at high efficiency (35%), whereas <0.1% of infected cells were found to have HCMV-TB40-BAC1 (Fig. 2a). This correlated well with a difference in the efficiency of nuclear translocation of virions after EC entry. When virus particles were visualized at 6 h after infection by indirect immunofluorescence against the capsid-associated HCMV-tegument protein pp150, only particles of HCMV-TB40-BAC4

\[ \text{Fig. 1.} \text{ Phenotype and genotype of viruses reconstituted from TB40/E-derived BAC clones. (a) Detection of viral IE antigens in HUVEC monolayers 7 days after co-culture of productively infected HFF with an excess of uninfected HUVEC indicator cells (ratio 1:3000). (b) Restriction fragment patterns of DNA from reconstituted TB40/E-BACs numbered 1, 3, 4 and 12. Arrowheads point to fragments differing between these clones.} \]

\[ \text{Fig. 2.} \text{ Phenotype of BAC clones 1 and 4 derived from HCMV TB40/E. (a) Infection efficiency of HCMV-TB40-BAC1 and HCMV-TB40-BAC4 in fibroblasts (HFF) and endothelial cells (HUVEC) was compared by detection of viral IE antigens (pUL122/123) 24 h after infection at an infection multiplicity of 0.7. Viral antigen was visualized by indirect immunofluorescence (Cy3, red nuclear signals). Counterstaining was done with DAPI (blue nuclear signals). (b) Nuclear localization efficiency of incoming virus was compared in HUVEC at 6 h after infection at an infection multiplicity of 5. Viral structural antigen pUL32 was visualized by indirect green immunofluorescence (punctate signals). Cellular tubulin was visualized by indirect red immunofluorescence (tubular signals). Counterstaining was done with DAPI (blue nuclear signals).} \]
translocated efficiently (>50%) towards the nucleus of infected HUVEC, whereas particles of HCMV-TB40-BAC1 remained in the periphery of the cell (nuclear translocation <1%, Fig. 2b). HCMV-TB40E-BAC12 behaved similarly to HCMV-TB40-BAC4 (data not shown), further corroborating the correlation between nuclear translocation and successful infection. Single-step growth curves showed that, apart from a slight delay in fibroblasts, HCMV-TB40-BAC4 greatly resembles the parental virus TB40/E with regard to release of high titres from both infected fibroblasts and EC as compared with the more cell-associated HCMV strain FIXBAC (Fig. 3).

**Fig. 3.** Single-step growth curves of HCMV-TB40/E, HCMV-TB40-BAC4 and HCMV-FIXBAC. Fibroblasts and EC were infected at an infection multiplicity of 1. Supernatant was collected daily from 3 to 7 days post-infection. The titre of infectious virus released by infected cell cultures was determined on fibroblast monolayers by limiting dilution analyses.

Interstrain differences in EC tropism between highly passaged HCMV strains and low passage HCMV strains have been linked to the genomic region UL128-UL131A. To analyse whether interstrain differences within this genomic region also determine cell tropism differences between HCMV-TB40-BAC1 and HCMV-TB40-BAC4, we compared the respective DNA sequences of these BACs. Fragments of the respective gene region were amplified from TB40-BAC1 and TB40-BAC4 using suitable primers, and the nucleic acid sequence was then determined and compared. Not a single base difference was found between the two BACs in UL130 and UL131A, whereas two single-nucleotide variations were detected in UL128. When compared to TB40-BAC4, TB40-BAC1 showed one adenine-to-cytosine exchange at nucleotide position 282 and an adenine insertion at nucleotide position 332 of the UL128 gene sequence (Fig. 4a). The base exchange A282C is located in the first intron and was therefore unlikely to alter the amino acid sequence of the respective protein. In contrast, the 332A insertion is located within the second exon and causes a frame shift resulting in a truncated pUL128 (Fig. 4b).

Next, we tested the hypothesis that the additional adenine at position 332 of UL128 is responsible for the loss of EC tropism of TB40-BAC1-derived viruses. We used the markerless ‘en passant’ mutagenesis previously published by Tischer et al. (2006) to remove the inserted adenine specifically from the TB40-BAC1 genome without leaving any further alteration (Fig. 5a). The obtained TB40-BAC1-UL128repair was reconstituted in fibroblasts, grown to high titres in HFF to avoid any selection for EC tropism, and then compared to HCMV-TB40-BAC1 and HCMV-TB40-BAC4 for infectivity in HUVEC. HUVEC and HFF were infected with all virus preparations at an m.o.i. of 1 p.f.u. per cell (normalized in HFF), and the ratio of infected cells was determined by immunofluorescent detection of IE antigen at 24 h after infection. The relative infection efficiency in HUVEC/HFF was 0.79 for HCMV-TB40-BAC4, 0.01 for HCMV-TB40-BAC1 and 0.84 for HCMV-TB40-BAC1-UL128repair (Fig. 5b and c). To further corroborate this finding, the reverse experiment was performed, inserting an adenine at position 332 of UL128 of TB40-BAC4. The reconstituted virus of this mutant had a relative infection efficiency in HUVEC/HFF of 0.01 (Fig. 5b and c), thus resembling HCMV-TB40-BAC1 which also carries the additional adenine.

Obviously, an insertion of adenine at position 332 of UL128 was the only coding difference between TB40-BAC4 and TB40-BAC1 in the previously described tropism-relevant gene region, and removal of this additional adenine was sufficient to restore EC tropism in the genomic background of TB40-BAC1.
Sequence alignment of TB40-BAC4 with various HCMV genomes

After successful generation of TB40/E-derived BACs and proof of their suitability for genetic modification by markerless removal or insertion of a single nucleotide in TB40-BAC1 or TB40-BAC4, respectively, we sought to obtain the genomic sequence and reading frame annotation of the highly endotheliotropic TB40-BAC4 as a basis for its future use by the scientific community. Sequencing was performed by a commercial service using a BAC DNA preparation that had been tested for its integrity by RFLA, virus reconstitution after transfection and phenotypical testing of the reconstituted virus in HUVEC.

From the obtained BAC sequence, non-HCMV sequences of the pEB1997 vector backbone were removed, and the sequence was positioned to start with the US segment adjacent to the BAC cassette (US7–US34), followed by the repeat regions and the UL segment. It is noteworthy that two neighbouring open reading frames (ORFs), IRS1 and US1, are missing in the TB40-BAC4 sequence. This is surprising because US1 and a part of US2 were used as the left flanking homology arm for the insertion of the BAC vector into the TB40/E genome. Indeed, a short part of US2 is present in TB40-BAC4, but US1 and IRS1 are absent. The reason for this is unknown, but it seems likely that it was caused by an illegitimate recombination event during the insertion of the BAC vector by homologous recombination in fibroblasts. Interestingly, a similar unanticipated deletion was found in the HCMV FIXBAC (Murphy et al., 2003b), which was constructed using the same BAC vector and flanking homologous arms (Hahn et al., 2002). With regard to previously reported TB40/E-variants, TB40-BAC4 resembles the Bart strain in that UL141 has a frameshift insertion at codon 63 (Tomasec et al., 2005); however, unlike strain Bart, UL144 and UL145 are intact and thus TB40-BAC4 is identical to the TB40/E sequence published by Dolan et al. (2004) in all three genes. The entire TB40-BAC4 sequence was annotated in analogy to other previously annotated HCMV strains and has been deposited under the designation TB40-BAC4 at the GenBank database (accession no. EF999921).

In order to get an idea about the relation of TB40-BAC4 to the other published HCMV genomes, sequences from eight HCMV strains were aligned using CLUSTAL W and similarity plots were performed comparing each genome with the consensus sequence obtained from the CLUSTAL W alignment. To enable an alignment, all genomes except TB40-BAC4 were transformed to prototype orientation in advance. Similarity plots showed that TB40-BAC4 is perfectly collinear with the HCMV ‘consensus sequence’ and has no gross deletions, except the US2-US6 region, which has been replaced by the BAC cassette and the adjacent IRS1-US1 region. A more detailed phylogenetic analysis of several glycoproteins showed that most of them can be individually classified into well defined genotypes except UL73 which is too polymorphic. TB40-BAC4 is closely related to FIXBAC in UL73, UL74, UL75, UL115 and UL120, closely related to Merlin in UL55 and UL119, closely related to AD169 in UL119 and UL120, closely related to TR-BAC in UL75 and UL119, closely related to PH-BAC in UL100 and closely related to Toledo in UL74 (Fig. 6).

Few genomic regions of TB40-BAC4 deviate from all other strains: RL6 is almost completely missing in TB40-BAC4, whereas it is present in all other strains. UL10 is highly divergent with an identity at the protein level of only 86.8% between TB40-BAC4 and AD169. pUL77 of TB40-BAC4 lacks 12 aa, resulting in an amino acid identity of 97.8%. pUL84 is 97.1% identical at the amino acid level including a 4 aa deletion in TB40-BAC4. pUL28 of TB40-BAC4 shows moderate divergence in the N-terminal half, resulting in 98% identity at the amino acid level.
Taken together, the information from the sequence comparison demonstrated that, apart from the replacement of the US1-US7 gene region by the BAC cassette, plus defects in RL6 and UL141, TB40-BAC4 appears to possess a relatively intact HCMV genome, which is collinear to all other strains and shares well defined glycoprotein genotypes.

DISCUSSION

The highly endotheliotropic strain TB40/E has been widely used for HCMV infection of EC, macrophages and dendritic cells. However, molecular analyses have been hindered by the fact that TB40/E was not available as a molecular clone. Furthermore, TB40/E was reported to be genetically heterogeneous (Dolan et al., 2004). By generation of TB40/E-derived BACs we have now solved both problems. TB40-BAC4 together with the genome sequence is now available for reconstitution of this highly endotheliotropic virus in a genetically pure and manipulable form.

The phenotypic and genotypic analysis of virus strains reconstituted from nine TB40/E-derived BACs confirmed the previous notion about heterogeneity of the parental virus. Heterogeneity was observed in restriction fragment patterns and cell tropism, though they did not correlate. A closer analysis of the UL128-UL131A genomic region revealed an additional nucleotide in the UL128 reading frame of TB40-BAC1, which leads to a frameshift, a truncated protein product and a loss of EC tropism. Thus, it is apparent that BAC cloning is suitable to obtain genetically defined virus clones even in cases where plaque purification failed to serve this purpose.

Identification of the subtle mutations underlying the cell tropism difference between RELA-identical clones HCMV-TB40-BAC1 and HCMV-TB40-BAC4 further confirmed previous reports, which demonstrated that loss of EC tropism during extended cell culture propagation is due to changes in the UL128-UL131A gene region (Akter et al., 2003; Adler et al., 2006; Hahn et al., 2004; Patrone et al., 2005; Wang & Shenk, 2005b). Targeted comparison of both BAC clones in the UL128-UL131A gene region revealed a single coding-relevant mutation only in UL128, and transfer of the UL128-BAC4 genotype into the background of TB40-BAC1 fully restored a high EC tropism in the parental virus.
HCMV-TB40-BAC1-UL128repair, which was indistinguishable from the phenotype of HCMV-TB40-BAC4. Vice versa, insertion of an adenine residue at the respective site in TB40-BAC4 destroyed its endotheliotropic phenotype. This is the first example illustrating restoration of the endotheliotropic phenotype in a poorly endotheliotropic HCMV UL128 mutant by orthotopic markerless repair of the respective gene, thus supporting previous findings with rescue of HCMV strain Merlin by transcomplementation (Hahn et al., 2004). As a recent sequence comparison of 34 clinical isolates did not detect any major alterations of the UL128 coding sequence such as frameshifts or premature stop codons among these isolates (Baldanti et al., 2006), the adenine insertion in TB40-BAC1 was most probably acquired during the initial propagation of TB40/E in fibroblast cultures. Obviously, although other gene regions were also reported to contribute to EC tropism as evidenced by loss of EC tropism after deletion of the respective ORF (Dunn et al., 2003; Pretsch et al., 2005), changes due to cell culture adaptation apparently target preferentially the UL128-UL131A gene region. An explanation for this is provided by recent reports on the inhibition of virus release of strain AD169 after repair of a defective UL131A or insertion of an intact UL131A ORF (Adler et al., 2006; Wang & Shenk, 2005a). Loss of the protein complex formed by gH/gL and the pUL128-131A gene products obviously provides a growth advantage in fibroblasts at the cost of a restricted cell tropism.

Maintenance of broad cell tropism, including EC tropism, is often at the cost of low titre virus production (Adler et al., 2006). HCMV-TB40-BAC4 is different as it combines both a high EC tropism and high titre virus release (Fig. 3), thus making it particularly suitable for applications where large amounts of virions are required, like electron microscopy or proteomic analyses of cell-free virus particles. The molecular determinants that allow for efficient release of infectious progeny while maintaining EC-tropism are widely unknown, albeit some hints may be derived from the whole genome comparison of TB40-BAC4 with other HCMV strains. One region where TB40-BAC4 differs from all other strains is the RL11 family. Particularly, TB40-BAC4 shows a loss of RL6 and a high degree of variation in UL10. Although some researchers have suggested RL6 may not be translated (Murphy et al., 2003a), this issue deserves further investigation. Alternatively, the minor deviation of TB40-BAC4 in UL28, UL77 and UL84 might also account for the unique features of this strain. The availability of TB40/E-derived BACs and the genome sequence of TB40-BAC4 should facilitate investigations of the contribution of these genes by the construction of the respective mutants.

In conclusion, we have generated a BAC derived from the HCMV strain TB40/E that combines the high titre growth of a cell culture-adapted strain with the broad cell tropism of a clinical HCMV isolate. Analysis of its genomic sequence showed a close relationship to the endotheliotropic strain FIXBAC, but also revealed that TB40-BAC4 shares the sequence of certain highly polymorphic genes with each of the other strains. The usability of TB40-derived BACs for genetic manipulations was proved by a marker transfer from TB40-BAC4 to TB40-BAC1 and vice versa, thus demonstrating the importance of the C-terminal part of pUL128 for EC tropism.

**Fig. 6.** Genomic comparison of TB40-BAC4 with other HCMV strains. Genotype trees obtained from phylogenetic analyses of the coding sequence of selected glycoproteins.
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