Spontaneous Activation of the Lytic Cycle in Cells Infected with a Recombinant Kaposi's Sarcoma-Associated Virus

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The genetic analysis of human herpesvirus 8 (HHV8), also termed Kaposi's sarcoma-associated virus, has been hampered by severe difficulties in producing infectious viral particles and modifying the viral genome. In this article, we report the successful cloning of the HHV8 complete genome onto a prokaryotic F-plasmid replicon which allows the propagation of the recombinant viral DNA in *Escherichia coli*. The insertion of the F-plasmid into the HHV8 genome interrupts the ORF56 gene, whose expression product—by homology with the Epstein-Barr virus BSLF1 gene—is supposed to be necessary for lytic DNA replication. After introduction of the recombinant HHV8 DNA into 293 cells, early viral antigens are expressed, suggesting that spontaneous lytic replication is initiated. However, completion of the lytic program is prevented by the absence of the ORF56 protein, and a quasi-latent state is established. Upon reintroduction of the ORF56 viral gene, the block is overcome and infectious HHV8 virions are produced. As the recombinant HHV8 genome can be easily modified in *E. coli*, this experimental system opens the way to an extensive genetic analysis of other HHV8 functions.

The discovery of the genome of a herpesvirus in cells from a Kaposi's sarcoma lesion was a major breakthrough in our understanding of the pathogenesis of this disease (21). This virus, termed Kaposi's sarcoma-associated herpesvirus or human herpesvirus 8 (HHV8), encodes a number of cytokines (macrophage-inhibitory protein, interleukin-6, and interferon regulatory factor), cyclin homologues, and G proteins that act as growth factors or promote cell proliferation. As a consequence, any of these gene products could potentially contribute to tumor cell growth (1, 3, 4, 19, 25). Viral inhibitors of apoptosis and viral proteins with transforming properties have also been identified, making this virus a strong candidate for the etiological agent of Kaposi's sarcoma and other virus-associated diseases (16, 26).

However, a number of basic questions concerning this virus are left unanswered. First, the target cell of HHV8 is unknown, and even the cell lineage of Kaposi's sarcoma is highly controversial. Second, the proteins and genomic elements that control the activation and completion of the viral lytic cycle are only partly understood. Finally, with the exception of a single report (11), all data concerning the transforming potential of this virus have been derived from experiments with single viral gene products, and nothing is known about the function of these gene products in the context of the whole virus. In particular, it is unclear whether all or only certain viral transforming proteins are required for cellular transformation. In this article, we report the successful cloning of the whole HHV8 genome in *Escherichia coli* as a recombinant F-factor-based plasmid which permits the generation of any viral mutant. We introduced the F-plasmid into ORF56 (21) of HHV8 strain

BC-3 (2), a gene that codes for a protein required for DNA replication, as indicated by its homology to the primase encoded by BSLF1 of Epstein-Barr virus (EBV) (10). Using this system, we show that cells carrying the recombinant HHV8 genome spontaneously initiate the viral lytic cycle, but that completion of the lytic cycle requires reintroduction of the ORF56 gene product.

MATERIALS AND METHODS

Cells. 293 is a human embryonic epithelial kidney cell line transformed by the E1a and E1b proteins from adenovirus strain 5 (13). This cell line was grown in RPMI 1640–10% fetal calf serum (Life Technologies, Eggenstein, Germany). The BC-3 cell line is a body cavity lymphoma cell line that was shown to carry HHV8 (2). This cell line was propagated in RPMI 1640–20% fetal calf serum.

Recombinant DNA plasmids. p1919 is an F-factor-based prokaryotic replicon that carries the F-factor origin of replication, the chloramphenicol resistance gene, the partitioning genes A and B, the hygromycin resistance cassette, and the gene that codes for the green fluorescent protein, as described previously (6). To provide the flanking regions for homologous recombination with the HHV8 genome, a DNA fragment (BC1 nucleotide coordinates 77407 to 87158) from the HHV8 genome BC1 (21) was introduced into plasmid pACYC177 cleaved with *NheI* to give p2388. The HHV8 genomic fragment was obtained after *SpeI* partial digestion of the GA21 HHV8 cosmid. p2388 encompasses ORF56 from HHV8, the homolog of the BSLF1 gene of EBV. The entire plasmid p1919 was introduced into the single *SpeI* site of p2388 to yield the final plasmid p2421 (see Fig. 1). p2421 was linearized with the *PspI* I restriction enzyme. The *AccI-XhoI* fragment from 2388 that contains HHV8 ORF56 was further subcloned onto the pKRV expression plasmid that carries a cytomegalovirus promoter (p2484).

DNA transfections. Transfections of cell lines with plasmid DNA were performed using lipid micelles or electroporation. For electroporation, BC-3 cells (10^7 cells) were washed in RPMI 1640 without fetal calf serum, resuspended in 250 μ l of the same medium, and placed with 10 μ g of p2421 plasmid DNA in 0.4-cm gap electroporation cuvettes. Cells were transfected using an electroporator (Bio-Rad Laboratories, Munich, Germany) at 230 V and 960 μ F. In order to introduce the recombinant HHV8 genome into a eukaryotic cell host 2×10^5 293 cells were incubated in Optimum minimal medium (Life Technologies, Eggenstein, Germany) for 4 h with 1 μ g of the recombinant HHV8/F-plasmid embedded in lipid micelles (Lipofectamine; Life Technologies). For completion of the viral lytic cycle, 1 μ g of p2484, which carries the ORF56 gene, was

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transfected into 2×10^5 293 cells, which stably carry the HHV8/F-plasmid recombinant virus.

Hygromycin selection. One day after transfection, BC3 cells were plated on 96-well cluster plates at a concentration of 10^4 cells per well, and hygromycin (Calbiochem, Munich, Germany) was added to the culture medium (300 μ g/ml). One day after infection or transfection of 293 cells in a six-well cluster plate, cells were expanded in a large culture plate (150 mm diameter), and selection was started at a 100 μ g of hygromycin per ml. Cells were fed weekly with fresh RPMI1640 with the same hygromycin concentration.

Plasmid rescue in *E. coli*. Circular DNA molecules were extracted from 10^7 293 cells stably carrying the HHV8/F-plasmid recombinant using a denaturation-renaturation method as described (14). *E. coli* strain DH10B was transformed with the extracted viral recombinant DNA by electroporation (1,800 V, 25 μ F, 100 Ω). Cells were plated on agar plates containing chloramphenicol (15 μ g/ml) for selection.

Infections. Infectious particles containing HHV8/F-plasmid DNA were obtained from 293 cells or BC3 cells stably carrying this construct and used to infect HHV8-negative 293 cells. Supernatants from 10^7 BC3/F cells (concentration of 10^6 cells per ml) in which the lytic cycle had been induced with tetradeconoyl phorbol acetate (TPA; 20 ng/ml final concentration) and butyrate (3 mM final concentration) for 3 days were used for infections (15, 17, 27). Similarly 5 ml of supernatants was obtained 3 days after transfection of 1 μ g of p2484 into 2×10^5 293-HHV8/F cells in one well of a six-well cluster plate. 293 cells (2×10^4) were infected with 1 ml of filtered (0.45- μ m pore size) infectious supernatants in a well from a 24-well cluster plate. In some cases, 293 cells were then selected for hygromycin resistance after expansion in large culture plates (150 mm diameter) and fed once a week with RPMI 1640 containing 10% fetal calf serum.

Southern blot analysis and Gardella gel analysis. The method for Gardella gel electrophoresis followed by Southern blot hybridization has been described previously (5, 12). We used 10 μ g of DNA for the Southern blot analysis and 10^6 cells per slot for the Gardella analysis. In both cases, a plasmid encompassing the F-plasmid or p2421 was radioactively labeled and used as a probe.

Immunostaining. Detection of the early antigen (ORF59) or of the K8.1A/B late HHV8 antigen in 293 cells carrying the HHV8/F-plasmid was performed using monoclonal antibodies specific to these proteins (Advanced Biotechnologies, Columbia, Mass.), as described previously (6).

RESULTS

Introduction of an F-plasmid into the HHV8 genome. A prerequisite for the manipulation of the HHV8 genome in *E. coli* cells is the introduction of a prokaryotic replicon into the viral genome. Since herpesviruses possess a very large genome, we chose the replicon of the F-plasmid, which is known to accept large DNA inserts and to replicate stably in *E. coli*. The genes encoding hygromycin resistance and green fluorescent protein were included to yield plasmid p1919 (6). In order to promote its homologous recombination with the viral genome, HHV8 flanking regions were added to the F-plasmid. We decided to insert the p1919 F-plasmid derivative into open reading frame 56 (ORF56) of HHV8. This gene is the homologue of the BSLF1 EBV gene that is indispensable for lytic viral DNA replication. As a consequence, the final viral mutant is incompetent to replicate, but its defect can be easily complemented. Towards this end, we constructed the final plasmid p2421, which carries the backbone of p1919 flanked by HHV8 sequences of about 4 and 5.7 kbp in length (BC1 coordinates 77407 to 81401 and 81402 to 87158) to target the ORF56 locus (Fig. 1). The linearized plasmid DNA fragment was then introduced into the BC-3 cell line, which harbors several extrachromosomal copies of the HHV8 genome. Cells were subjected to hygromycin selection (300 μ g/ml) after plating in 96-well cluster plates.

Four weeks after selection, more than 40 hygromycin-resistant cell clones positive for green fluorescent protein were established and characterized by Southern blot analysis. One cell clone, termed BC-3/F, was found to carry the F-plasmid

FIG. 1. Schematic representation of the construct used for homologous recombination. A plasmid backbone consisting of the F-plasmid replicon, the hygromycin resistance gene, and the gene for green fluorescent protein (gfp) was flanked by HHV8 sequences situated left and right from the *SpeI* site of the ORF56 gene. Successful homologous recombination inserts the F-plasmid, the hygromycin resistance gene, and the green fluorescent protein gene into the ORF56 gene locus, leading to a knockout mutation of this gene. As we used cosmids from HHV8 strain BC1 to generate the flanking sequences and because this strain is known to show some sequence variation from the BC-3 virus, we sequenced the entire 8-kb region around the *SpeI* site contained in the ORF56 gene. The sequencing showed only four minor variations compared to the published sequence (21) (data not shown).

inserted correctly into the HHV8 genome (Fig. 2). Cell culture supernatant was harvested from this cell clone after incubation with TPA and butyrate. The supernatant contained infectious virions, as indicated by the expression of green fluorescent protein after infection of 293 cells (data not shown). After selection with hygromycin (100 μ g/ml), seven 293 cell clones that carried the viral genome were obtained, as shown by a Gardella gel analysis (Fig. 3). None of these 293-HHV8/F cell clones spontaneously produced virions, as expected from the insertional mutagenesis of the ORF56 gene. Southern blot analysis of one of these 293 HHV8/F cell clones (293 HHV8/F III) showed that it contained recombinant plasmids only, excluding a coinfection with wild-type HHV8 (Fig. 2).

Recovery of recombinant virus genome in *E. coli*. The 293-HHV8/F cell clones infected with supernatant from the BC-3/F cell line and selected for hygromycin resistance contained extrachromosomal copies of the recombinant HHV8 genome, as shown in Fig. 3. Rescue of these circular molecules from one of these clones (293 HHV8/F III) gave rise to chloramphenicol-resistant *E. coli* cell clones that proved to contain the HHV8/F plasmid hybrid (Fig. 4). Comparison of several restriction enzyme DNA fragment patterns with those deduced from the analysis of the published genomic HHV8 sequences (21) unambiguously identified the rescued genome as being the complete genome of HHV8. In a further step, we constructed an *SpeI* library from the cloned HHV8/F-plasmid genome, which proved to contain all *SpeI* fragments expected from the available sequence (viral strain BC1). These clones themselves contained all expected *Bam*HI restriction sites. Partial sequencing of certain *SpeI* subclones confirmed the successful cloning of the BC3 genome in *E. coli* (data not shown). However, analysis

FIG. 2. Southern blot analysis of bacterial and eukaryotic cellular DNAs containing the recombinant HHV8/F-plasmid. (A) Schematic representation of the expected restriction map after *Bam*HI digestion of wild-type HHV8 DNA (HHV8 BC1), p2421 plasmid that was used for recombination, and recombinant HHV8/F-plasmid. The maps are deduced from the published HHV8 sequence (BC1 strain). The sizes of the expected fragments are indicated (in base pairs). (B) DNAs extracted from bacterial cells containing HHV8/F-plasmid DNA, hygromycin-resistant BC3 clone (BC3/F), or 293 cells stably carrying the HHV8/F-plasmid after transfection (293 HHV8/F III) or infection (293-T-HHV8/F VIII) were digested with *Bam*HI and separated on a 0.8% agarose gel. DNAs extracted from the BC3 cell line and the p2421 plasmid were used as positive controls. After blotting, the DNAs were hybridized with ³²P-radiolabeled p2421 DNA. The 4.7-kb signal is characteristic of wild-type HHV8 DNA and is disrupted by the recombination, whereas the 2- and 12.1-kb fragments are generated by the recombination. This Southern blot analysis shows that recombination took place as expected and that the 293 HHV8/F III and 293-T-HHV8/F VIII cell clones contain only the recombinant HHV8/F-plasmid. The 14.9-kb band, expected in all samples, is only partly identified by the 2421 probe, leading to weaker signals. The p2421 plasmid contains only part of the *Bam*HI 3.9-kb fragment from the HHV8 genome, (3.6 kb), as well as an additional 9-kb DNA fragment in the BC3 cell lines that is absent from the recombinant viruses. This fragment is not expected from the HHV8 sequence and possibly stems from a modified episome subpopulation in the BC3 cell line.

FIG. 3. Gardella gel analysis of hygromycin-resistant 293 cell clones. 293 cells were infected with supernatants from induced BC3 cell clones that contain the recombinant HHV8/F-plasmid. After hygromycin selection, seven clones were analyzed for the presence of circular molecules that carry the F-plasmid. After hybridization with a probe specific for the F-plasmid, all cell lines proved to carry the recombinant virus genome. No lytic replication could be definitely assessed, and the faint signals observed most probably correspond to nonspecific DNA degradation products that happen to migrate at the same position. However, the origin of this signal cannot be definitely assessed, as indicated by the question mark.

of the recombinant viral genome with additional restriction enzymes showed occasional divergence from the predictions obtained with the BC1 HHV8 sequence. Minor variation in sequences is expected from two different viral strains.

Stable transfection of recombinant HHV8/F plasmid into 293 cells. In order to ensure that the HHV8/F-plasmid carried the complete viral genome and that passaging of the viral DNA in *E. coli* did not alter the ability of HHV8 to replicate and give rise to progeny virus (see below), 293 cells were stably transfected with the recombinant HHV8/F-plasmid and selected with hygromycin. Eight clones, termed 293-T-HHV8/F cells, were isolated. The Southern blot analysis of one of these clones showed that it carries the HHV8/F plasmid in the absence of wild-type viral DNA, as expected (293-T-HHV8/F VIII) (Fig. 2).

293 HHV8/F cells spontaneously express the HHV8 ORF59 early antigen. Some members of the herpesvirus family, like herpes simplex virus type 1, spontaneously enter the lytic cycle after infection of their target cells. Propagation of the virus from one cell to another results in rapid multiplication and a dramatic increase in virus titer. However, for gammaherpesviruses like EBV, spontaneous lytic replication rarely occurs in vitro and latent infection is the rule. Although cell lines derived from tumor biopsies are predominantly latently infected with HHV8, it does not necessarily mean that 293 cells can also carry the wild-type HHV8 in a latent form. However, as the HHV8 recombinant virus has a mutated ORF56 gene, lytic replication cannot proceed. ORF56 encodes the homologue of the EBV primase, which is considered an early gene product and indispensable for herpesvirus DNA lytic replication. The

immediate-early and early stages of the EBV lytic program are not dependent on the expression of BSLF1. If the parallel between BSLF1 and ORF56 is correct, spontaneous activation of the HHV8 lytic program in 293 cells should lead to expression of early HHV8 genes upstream of ORF56. Using an antibody directed against the ORF59 antigen, the homologue of the EBV early antigen BMRF1, 30% of the 293 HHV8/F

FIG. 4. Restriction analysis of recombinant HHV8 DNA. Circular molecules were extracted from the hygromycin-resistant 293-HHV8/F III cell clone that was identified as containing the entire HHV8/F-plasmid. After electroporation into *E. coli* strain DH10B and chloramphenicol selection, plasmid DNA was extracted and digested with *XhoI* and *BamHI*.

FIG. 5. 293 cells that carry the recombinant HHV8/F-plasmid spontaneously enter the lytic cycle. 293-T-HHV8/F cells and 293-HHV8/F cells were stained with an antibody specific for the early ORF59 protein. Fixed cells were incubated with a specific monoclonal antibody and a second anti-mouse immunoglobulin antibody coupled to the indocarbocyanine fluorochrome. Stained cells were visualized under UV light. Magnification, $\times 100$.

and of the 293-T-HHV8/F cell clones were found to express this viral protein (Fig. 5). This finding indicates that the initial events in the HHV8 lytic gene expression cascade take place spontaneously in 293 cells.

Transfection of ORF56 gene product leads to production of infectious virions in 293 cells. Next, we wished to test the ability of both the 293 HHV8/F III and 293-T-HHV8/F VIII cell lines to support the complete lytic phase of HHV8's life cycle. Transient transfection of an expression plasmid encompassing the ORF56 gene into these cells led to the production of infectious particles. Supernatants derived from transfected cells were able to infect parental 293 cells, as indicated by the expression of green fluorescent protein observed 3 days after infection (Fig. 6). Comparable virus stocks could be obtained from both cell lines, 293-HHV8/F III and 293-T-HHV8/F VIII. The number of cells which were positive for green fluorescent protein upon infection was 102 out of 10^5 infected 293 cells (average of three sets of experiments). This is in the same range as what we observed with supernatants containing 10^5 EBV infectious particles per ml (6). It is difficult to evaluate the virus titers in these supernatants because the efficiency with which 293 cells can be infected with HHV8 is unknown (20). In conclusion, the recombinant virus proved to be fully functional in terms of production of infectious particles, indicating that propagation of the complete HHV8 genome in *E. coli* preserved the viral functions, very similar to the situation with EBV (6). However, after more than 2 months of continuous culture, the ability of the 293-HHV8/F III and 293-T-HHV8/F VIII cell clones to sustain viral lytic replication decreased. This effect is probably linked to gradual silencing of viral promoters, as incubation of these cells with butyrate in combination with transfection of the p2484 expression plasmid led to the expression of the K8.1A/B late gene product (Fig. 7). Butyrate is a

potent inhibitor of histone deacetylase, causing transcriptional repression by chromatin condensation.

DISCUSSION

The genetic analysis of a given organism, i.e., the construction of viral mutants, is the most straightforward way to understand the function(s) of a given gene in the context of the entire genome. However, the practical realization of these mutants is often quite tedious. Although the genetic complexity of herpesviruses is far from that encountered in mammalian genomes, the relatively large size of herpesvirus genomes prevents their conventional cloning. The production of infectious particles is a recurrent problem when working with EBV or HHV8. Until now, no cellular system has supported their propagation *in vitro*. This renders the construction of viral mutants much more difficult than with other herpesviruses. In the case of HHV8, even the identification of infected cells is difficult and often relies on PCR detection of spliced products (20). The normal counterpart of the Kaposi's sarcoma spindle cells is still unknown, although they may be related to the endothelial cells of the vascular or lymphatic system (8).

As an attempt to circumvent these problems, we have cloned the whole viral genome onto an F-plasmid that carries the green fluorescent protein phenotypic marker. This should prove to be helpful for the identification of HHV8 target cells. The recombinant virus includes all known sequences, as shown by restriction analysis and subcloning. By inserting the F-plasmid into ORF56, we interrupted a gene whose EBV homologue is involved in the viral lytic replication machinery, hoping to obtain an inducible system. As anticipated, transient transfection of a plasmid encompassing the disrupted ORF56 gene is able to complement the defective mutant and suffices to

293-HHV8/F supernatant

A

293-T-HHV8/F supernatant

B

FIG. 6. Infection of 293 cells with recombinant HHV8/F-plasmid virus. The 293 HHV8/F III cell line (A) and the 293-T-HHV8/F VIII cell line (B) were transfected with ORF56 cloned onto an expression plasmid. After 3 days, the supernatants from these transfected cell lines were incubated with HHV8-negative 293 cells. After 3 days green fluorescent protein-positive, HHV8-infected 293 cells could be observed. Magnification, $\times 100$.

complete the viral lytic cycle, confirming that the recombinant virus carries the complete HHV8 genome. Although the ORF56 gene shows homologies to the EBV protein BSLF1, it cannot be characterized as an immediate-early gene like BZLF1 in EBV (23). However, the ORF56 gene has similar functions from a practical point of view. The ability of ORF56 to complement the viral phenotype implies that the 293 cells that carry the recombinant plasmid spontaneously express

some early viral antigens. In fact, immunostaining using an antibody against the ORF59 early antigen proved that some of the HHV8-positive cells spontaneously entered the lytic cycle.

The observation that expression of the ORF56 protein leads to completion of the viral lytic cycle is apparently contradictory to a report which claims that infection of 293 cells with wild-type HHV8 leads to an abortive lytic viral replication (20). In fact, cells infected with the HHV8/F-plasmid recombinant did

293-HHV8/F III

α -K8.1 A/B

FIG. 7. Expression of K8.1 A/B late viral antigen in 293-HHV8/F III cells. The 293 HHV8/F III cell line was transfected with ORF56 cloned onto an expression plasmid and simultaneously incubated with 3 mM sodium butyrate. After 3 days in culture, cells were stained with an antibody specific to the late K8.1 A/B viral protein. Fixed cells were incubated with a specific monoclonal antibody to this antigen and a second anti-mouse immunoglobulin antibody coupled to the indocarbocyanine fluorochrome. Stained cells were visualized under UV light. Magnification, $\times 100$.

not produce any progeny, as attested by the absence of propagation in infected cultures. At this stage, it is only possible to speculate that HHV8 lytic replication must be preceded by a phase of latent infection. However, the molecular mechanisms that control this transition are still unknown. A similar situation is observed with EBV, with which cell infection is rarely followed by spontaneous replication, whereas some cells latently infected with EBV can be induced to produce virions.

The cloning of a complete herpesvirus genome has been valuable for the modification of several herpesvirus genomes (6, 9, 18, 22, 24). Even deleterious mutations are possible, as we have demonstrated recently in order to construct the first helper cell line for the encapsidation of EBV-derived viral vectors (7). As HHV8 can immortalize endothelial cells in vitro, it is now theoretically possible to generate viral mutants that have deletions of one or several genes thought to be involved in cell transformation (11). Most interesting, the function of a single gene can be studied in the context of the whole genome. The identification of the gene products that mediate the transforming potential of HHV8 will allow the construction of apathogenic strains that are of potential interest for vaccination of individuals who are at high risk for the development of Kaposi's sarcoma.

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