The putative U94 integrase is dispensable for human herpesvirus 6 (HHV-6) chromosomal integration

Nina Wallaschek,1 Annie Gravel,2 Louis Flamand2,3 and Benedikt B. Kaufer1

1Institut für Virologie, Freie Universität Berlin, Robert von Ostertag-Straße 7-13, 14163 Berlin, Germany
2Division of Infectious Disease and Immunity, CHU de Québec Research Center, Quebec city, Quebec G1V 4G2, Canada
3Department of Microbiology, Infectious Disease and Immunology, Faculty of Medicine, Université Laval, Quebec city, Québec, G1V 0A6, Canada

Human herpesvirus 6 (HHV-6) can integrate its genome into the telomeres of host chromosomes and is present in the germline of about 1% of the human population. HHV-6 encodes a putative integrase U94 that possesses all molecular functions required for recombination including DNA-binding, ATPase, helicase and nuclease activity, and was hypothesized by many researchers to facilitate integration ever since the discovery of HHV-6 integration. However, analysis of U94 in the virus context has been hampered by the lack of reverse-genetic systems and efficient integration assays. Here, we addressed the role of U94 and the cellular recombinase Rad51 in HHV-6 integration. Surprisingly, we could demonstrate that HHV-6 efficiently integrated in the absence of U94 using a new quantitative integration assay. Additional inhibition of the cellular recombinase Rad51 had only a minor impact on virus integration. Our results shed light on this complex integration mechanism that includes factors beyond U94 and Rad51.

Human herpesvirus-6A and -6B (HHV-6) are closely related betaherpesviruses that infect humans early in life (Salahuddin et al., 1986). HHV-6B is the causative agent of roseola infantum, a febrile illness in infants, while disease associations of HHV-6A are not well characterized (Yamanishi et al., 1988). Both viruses have previously been shown to integrate their genetic material into telomeres of human chromosomes (Arbuckle et al., 2010, 2013). This integration mechanism also allows vertical transmission of the virus via the germline, resulting in individuals that harbour the integrated virus in every single cell of their body (Daibata et al., 1998, 1999; Tanaka-Taya et al., 2004; Mori et al., 2009; Kühl, et al., 2014; Osterrieder et al., 2014). This condition is termed inherited chromosomally integrated HHV-6 (iciHHV-6) and is present in about 1% of the human population (Pellett et al., 2012). The biological consequences of this condition are not yet well understood. A recent report indicates that iciHHV-6+ subjects are at greater risk of developing angina (Gravel et al., 2015). The molecular mechanism and the factors involved in HHV-6 integration remain completely unknown.

HHV-6 encodes the U94 gene that contains all conserved domains of the Rep68 integrase of Adeno-associated virus 2 (AAV-2) (Thomson et al., 1991; Arbuckle & Medveczky, 2011). Expression of U94 restores replication of a Rep-deficient AAV-2, suggesting that both proteins have similar functions (Thomson et al., 1994). U94 of both HHV-6A and HHV-6B has been shown to possess single-strand and double-strand DNA-binding activity with a preference for TTAGGG repeats, as present in human telomeres. In addition, U94 has ATPase, helicase and 3’–5’ exonuclease activity against 3’ recessive ends in vitro (Trempe et al., 2015). To date, the role of U94 in virus replication and integration could not be determined due to the lack of reverse-genetic systems and efficient integration assays that would allow quantification of virus integration.

To investigate the role of U94 in HHV-6 integration, we deleted the entire U94 gene (ΔU94) in pHHV-6A (wt), a recently generated infectious bacterial artificial chromosome (BAC) clone of HHV-6A (strain U1102), using two-step Red-mediated mutagenesis (Fig. 1a) as previously described (Tischer et al., 2006; Tang et al., 2010; Tischer &...
Kaufer, 2012). In addition, we generated a revertant virus in which we restored U94 to ensure that no secondary mutations occurred during the mutagenesis process. Primers used for the mutagenesis are listed in Table S1 (available in the online Supplementary Material). Recombinant BAC clones were confirmed by RFLP, DNA sequencing and Southern blotting using a specific digoxigenin (DIG)-labelled probe for U94 (Fig. 1b) as described previously (Kaufer et al., 2011). The recombiant viruses were subsequently reconstituted by nucleofection of JJHan cells with the HHV-6A BAC DNA as described previously (Nukui et al., 2015). Cell-free virus stocks were generated by concentrating the supernatant of highly infected JJHan cells and titrated by analysing the genome copies in newly infected cells by qPCR. Multi-step growth kinetics (Oyaizu et al., 2012; Nukui et al., 2015) revealed that the ∆U94 mutant had a significant growth defect compared to the wt and revertant virus (Fig. 1c), suggesting that the impaired HHV-6A replication is either due to the abrogation of U94 expression or effects on neighbouring genes such as U95 caused by deletion of the U94 sequences.

To determine the integration efficiency of recombinant HHV-6A viruses, we used our recently established integration assay (Gravel and others, unpublished). Briefly, U2OS cells were infected with wt, ∆U94 and ∆U94rev that express GFP under the control of the HCMV major immediate early promoter. Infected GFP-positive cells were isolated 36 h post-infection using a FACS AriaIII cell sorter (BD), providing a pure infected cell population for our integration assays. To determine the integration efficiency of respective viruses, we performed fluorescence in situ hybridization (FISH) analyses on the infected U2OS cultures at day 14 post-sorting as described previously (Kaufer et al., 2011; Kaufer, 2013). Despite the growth defect of ∆U94 during lytic replication, the integration frequency of ∆U94 in metaphase chromosomes was comparable to wt and revertant virus (Fig. 2a, b); for this purpose 90 metaphases for each virus were imaged and the proportion (%) that showed a specific signal for HHV-6 at the ends of chromosomes in two chromatids was determined. To confirm that this is not a metaphase-specific effect, we analysed interphase nuclei for the presence of the virus genome. ∆U94 was detected in comparable proportions of nuclei (Fig. 2c). These findings indicate that U94 is not essential for HHV-6A integration. Furthermore, we performed qPCR analyses at day 0 and day 14 post-sorting to determine whether the maintenance of the HHV-6A genome was altered upon abrogation of U94. Viral genome copies were quantified by qPCR relative to the cellular genome, using primers and probes specific for HHV-6A U86 and the cellular β2M gene, respectively (Table S1). The HHV-6A genome was maintained in ∆U94-infected cells at comparable levels to wt and revertant viruses (Fig. 2d). Furthermore, clonal cell lines for wt, ∆U94 and ∆U94rev were generated to confirm the integration into host telomeres (Fig. 3).

To exclude that the alternative lengthening of telomeres (ALT) activity present in U2OS cells can compensate for the absence of U94, we confirmed our observations in ALT-negative 293T cells (Fig. S1) and JJHan cells (data not shown). Furthermore, no integration defect was observed in a HHV-6A mutant virus in which U94 expression was abrogated by insertion of multiple stop codons within the open reading frame (Fig. S2), while viruses that lacked the viral telomere sequences had a severe integration defect (Wallaschek et al., 2016). Taken together, our data demonstrate that U94 is not essential for HHV-6A integration in U2OS, 293T and JJHan cells.

Next, we investigated whether the cellular recombinase Rad51 that is essential for homologous recombination in mitotic cells (San Filippo et al., 2008) is involved in the integration of HHV-6A and could facilitate integration in the absence of U94. Therefore, we performed in vitro integration assays with wt and ∆U94 mutant viruses in the presence or absence of the specific Rad51 inhibitor RI-1. We used 5 μM of RI-1 as this concentration was previously shown to efficiently inhibit Rad51 (Budke et al., 2012), while no cytotoxic effects were detectable (Fig. S3). Both the wt and ∆U94 mutant were still able to integrate into U2OS cells, and although a slightly decreased frequency in metaphases and interphases was observed in the presence of RI-1 (Fig. 2e, f), our data suggest that Rad51 does not play a
major role in the integration of HHV-6A in U2OS cells. Comparable results were obtained with the commercial Rad51 inhibitor B02 (data not shown). Similarly, qPCR analyses revealed a minor reduction in HHV-6A genome copies in RI-1-treated cells (Fig. 2g). Taken together, our data demonstrate that the putative viral integrase U94 and the cellular recombinase Rad51 are both dispensable for the integration of HHV-6A into host chromosomes in the tested cell lines, suggesting that other viral or cellular recombinases can complement their functions. Most likely,
several redundant pathways ensure that HHV-6 integration can occur. Besides homologous recombination, single-strand annealing is a mechanism that could potentially accomplish virus integration. In this scenario, cellular factors such as Rad52 or viral factors including U41 and U70, the homologues of HSV-1 UL12 and ICP8, which were shown to mediate strand exchange (Reuven et al., 2003; Schumacher et al., 2012), could also facilitate HHV-6 integration and are targets of future studies. In addition, we will establish a quantitative integration assay for primary cells to assess the role of U94 in cells ex vivo.

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