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Chromosomally integrated HHV-6: impact on virus, cell and organismal biology

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HHV-6 integrates its genome into telomeres of human chromosomes. Integration can occur in somatic cells or gametes, the latter leading to individuals harboring the HHV-6 genome in every cell. This condition is transmitted to descendants and referred to as inherited chromosomally integrated human herpesvirus 6 (iciHHV-6). Although integration can occur in different chromosomes, it invariably takes place in the telomere region. This integration mechanism represents a way to maintain the virus genome during latency, which is so far unique amongst human herpesviruses. Recent work provides evidence that the integrated HHV-6 genome can be mobilized from the host chromosome, resulting in the onset of disease. Details on required structural determinants, putative integration mechanisms and biological and medical consequences of iciHHV-6 are discussed.

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Introduction

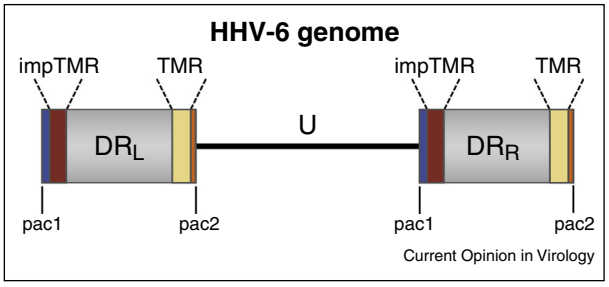
The genomes of human herpesvirus 6A (HHV-6A) and HHV-6B consist of a single unique segment (U) (~145 kbp) flanked by identical direct repeats (DR) (~9kbp) [1–4]. The DRs are flanked by pac1 and pac2 sequences that are involved in the cleavage and the packaging of the HHV-6 genome (Figure 1) [5,6]. Adjacent to the pac2 sequences is an array of telomeric repeats (TMR) that are identical to the human telomere sequences (TTAGGG). In proximity to pac1 is a second telomere array, consisting of TMR that are disrupted by other repetitive sequences, termed imperfect TMR (impTMR) [3,6]. Intriguingly, TMR are found in several lymphotropic herpesviruses belonging to the *alpha*, *beta and gammaherpesvirinae* [1,3,7–12] as discussed in greater detail below.

Although HHV-6A and HHV6B viral genome integration occurs in several distinct chromosomes, it invariably takes place within the telomeric region of the host chromosomes. The precise mechanism that facilitates integration is still to be defined; however, the presence of TMR at the ends of the viral genomes suggests that these sequences are involved by directing integration into host telomeres. A role for the U94 protein in HHV-6A and HHV-6B integration has been proposed, but remains to be proven experimentally. The relatively wide tropism of HHV-6A and HHV-6B suggest that integration can take place in many different cell types, including gametes. Integration into gametes results in individuals carrying a copy of the HHV-6 genome in every cell of their body. This condition is referred to as inherited chromosomally integrated HHV-6 (iciHHV-6) and is quite common as it is observed in approximately 1% of the human population (50–70 million individuals) worldwide. iciHHV-6 should be distinguished from the commonly used term ciHHV-6 that refers to the presence of integrated HHV-6 genomes, regardless if this is inherited or not. Individuals with iciHHV-6 will transmit the integrated HHV-6 genome according to the Mendelian laws, meaning that 50% of the descendants will inherit iciHHV-6. In this review, the biology of viral integration, the possible medical consequences associated with iciHHV-6 and priority research areas will be discussed.

What is known about HHV-6 latency? Is integration the default mechanism for genome maintenance during latency for HHV-6?

One hallmark of all herpesviruses is that they not only replicate in the infected host but also establish a lifelong persistent infection termed latency. Latency is characterized by the continued presence of the viral genome in infected target cells but the absence of infectious virus production. HHV-6 has been shown to establish a latent infection in various cell types including early bone marrow progenitor cells [13], primary monocytes/macrophages [14], myeloid cell lines [15], an astrocytoma cell line [16] and an oligodendrocyte cell line [17]. In most of these cell types, HHV-6 genes involved in lytic replication are not express and the virus can reactivate, suggesting that it is a quiescent rather than an abortive infection. The target cells differ between HHV-6A and HHV-6B, but more work needs to be done to define the true latency reservoir of both viruses.

Figure 1

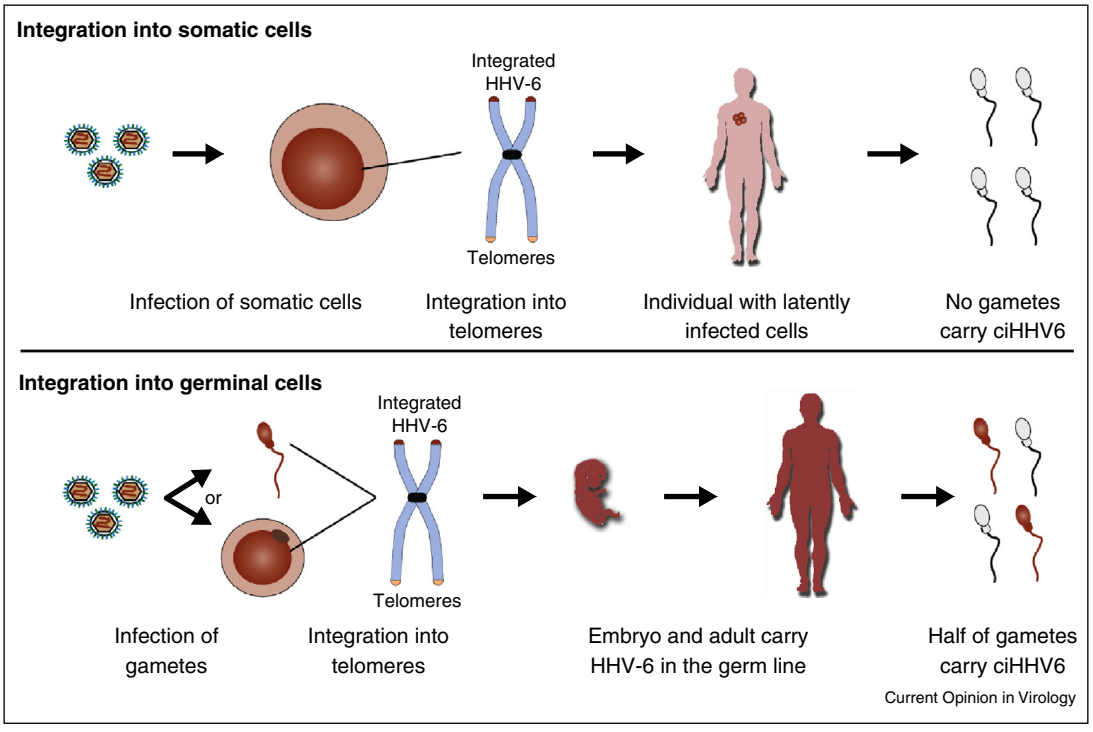


Schematic representation of the HHV-6 genome. The unique region (U) of the HHV-6 genome (140 kbp) is flanked by two identical direct repeat sequences (10–13 kbp) referred to as DR_L and DR_R. The DRs contain pac1 and pac2 sequences, perfect (TMR) and imperfect (impTMR) telomeric sequences (TMR) and several open reading frames (not shown). The genome is not drawn to scale.

During latency, a limited number of transcripts are expressed. Four latency-associated transcripts encoded from the HHV-6 IE1/IE2 locus, are highly spliced and only expressed in latently infected cells *in vitro* and *in vivo* [18]. It has been proposed that these transcripts give rise to three latency-associated proteins termed ORF99, ORF142, and ORF145; however no experimental evidence that confirms their expression is available yet. Furthermore, U94 has been shown to be expressed during latency, even at higher levels compared to lytically infected cells [19]. The U94 protein also blocks viral gene expression in infected lymphocytes in culture [19,20], suggesting that U94 is involved in the establishment and/or maintenance of latency.

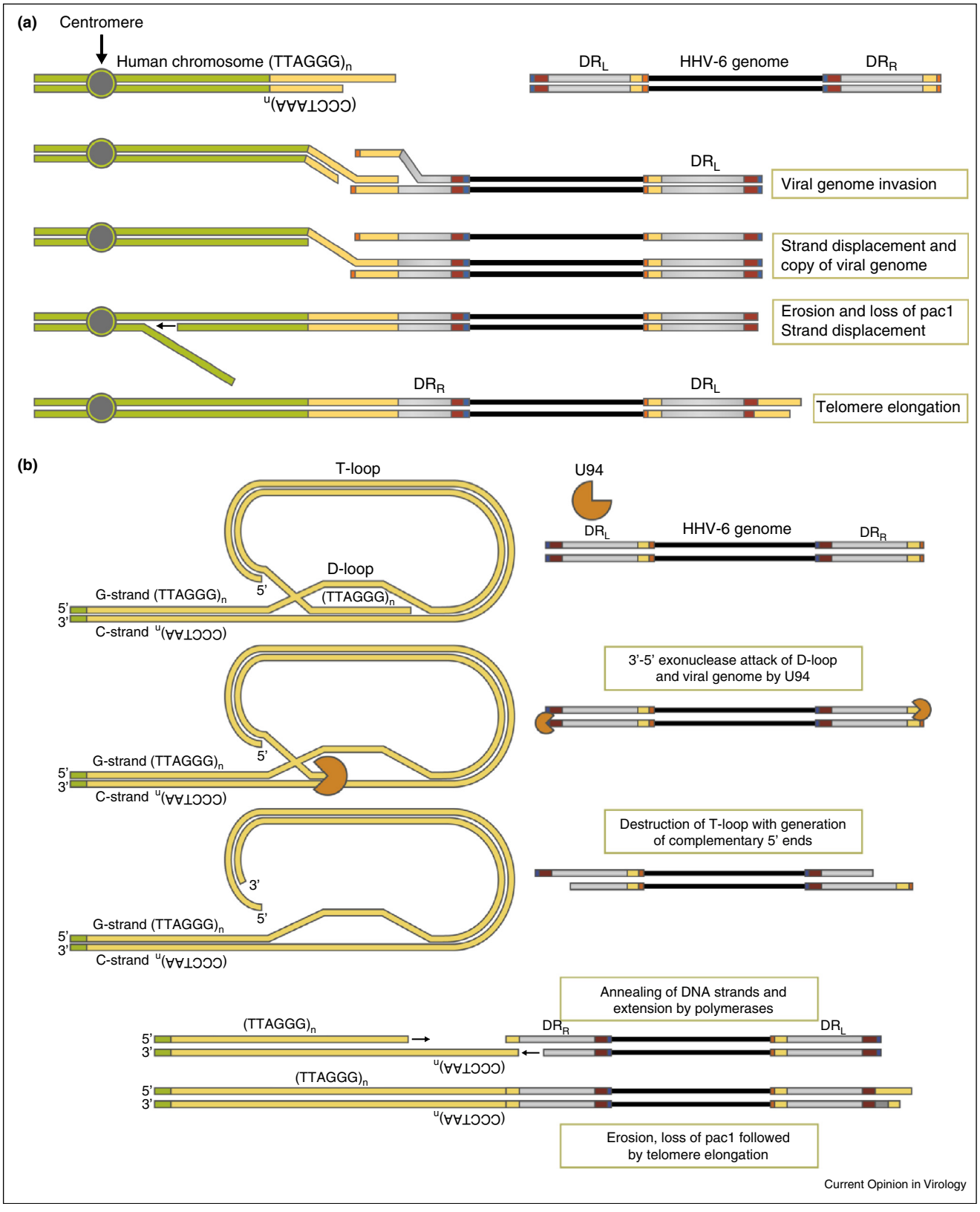
As mentioned above, HHV-6 has been shown to integrate its genome into host telomeres of latently infected cells (Figure 2). Integration of HHV-6 also occurs upon infection of various cell lines including JJhan and Molt-3 T-cells [21••,22], the human embryonic kidney cell line HEK293 [21••,23]. Integration of HHV-6 is not a dead end, as virus reactivation can be induced in cells that harbor the integrated virus genome using the HDAC inhibitor trichostatin A (TSA) or tetradecanoylphorbol-acetate (TPA) [14]. While most herpesviruses maintain their genome as a circular episome in latently infected cells, no episomal copies of the HHV-6 genome were detected [21••,22]. Since only the integrated form of the HHV-6 genome is present during latency, it is likely that integration is the default mechanism for genome maintenance in this phase

Figure 2



Integration of HHV-6 into chromosomes of somatic and germinal cells. During primary HHV-6 infection early in life, the virus infects somatic cells such as monocytes, macrophages and T-cells (upper panel). HHV-6 is able to integrate its genome and establish latency in these cells. Some latently infected cells remain in the host for life, but the virus is not transmitted via the germ line. In addition, HHV-6 is able to infect germinal cells (lower panel). Integration of HHV-6 into sperm cells or oocytes can result in an individual that harbors the virus in the germ line. These individuals pass on iciHHV-6 to 50% of their offspring according to the Mendelian laws of inheritance.

Figure 3



Hypothetical models leading to HHV-6 genome integration into host chromosomes. **(a)** A model based on break-induced homologous recombination (BIR) would allow invasion of 3′ end of the chromosome into the viral genome at the DR_R TMR, followed by stand displacement and copying of the viral

of infection; however, further work needs to be done to determine whether latency can be achieved without integration, i.e. as a viral episome, to decipher the integration mechanism and to understand how the virus genome is mobilized during reactivation.

What is the underlying mechanism of integration? Which cellular and viral factors are potentially involved in the process?

The termini of eukaryotic chromosomes consist of conserved structures termed telomeres that protect the genetic information from terminal deterioration. Vertebrate telomeres consist of 7–10 kb hexameric repeats $(TTAGGG)_n$ that are associated with a number of proteins. During DNA replication, the terminal portion of the telomeres is not completely copied due to the end replication problem. To counteract this shortening, certain cell types express the telomerase complex, which extends the telomeres by the addition TMR sequences to the terminus.

Intriguingly, the HHV-6A and HHV-6B genomes contain TMR at their termini (Figure 1). The fact that HHV-6A and HHV-6B integration invariably occurs in the telomeric region of human chromosomes, suggest that homologous recombination (HR) events between host and viral TMR could facilitate integration.

Chromosome ends have a 3′ single-stranded G-rich (TTAGGG) overhang that is 30–500 nucleotides in length [24–26]. To avoid recognition as double-stranded DNA (dsDNA) break, the 3′ protruding end folds back and invades the duplex telomeric DNA to generate a T-loop structure [27–29]. A total of six proteins referred to as the shelterin complex, bind and assist with T-loop formation, stabilize chromosomal ends and prevent DNA damage responses [30]. Sequencing of the HHV-6 integration sites indicated that the DR_R region of the viral genome is fused to the chromosome [31••]. The pac2 region at this extremity is lost during the integration process. In addition, the pac1 sequences at the other end of the viral genome (DR_L) are also lost and the viral TMR are extended with TTAGGG repeats [31••].

One model for HHV-6 integration compatible with this structure is based on the DNA repair mechanism referred to as break-induced replication (BIR). BIR is a HR pathway that facilitates the repair of DNA breaks that have only one end, contributing to the repair of broken replication forks and allowing telomere lengthening in the absence of telomerase. BIR has been described in various organisms including viruses, bacteria, and eukaryotes (reviewed in [32]). In the context of HHV-6 integration, BIR could be initiated by invasion of the 3′ single strand chromosomal end into the dsDNA linear HHV-6 genome at the TMR sites, followed by DNA synthesis that would continue to the end of the viral genome (Figure 3a). Upon cell replication and division, the 26–28 nucleotide pac1 sequences could erode until the TMR region is encountered. TMR could then serve as template for telomerase, as recently reported [33]. It remains unknown which cellular/viral proteins could participate in this BIR-dependent HHV-6 integration. Considering that BIR is a process that can occur independently of infection, one would argue that viral proteins are dispensable. If HHV-6 integration occurs through BIR, other herpesviruses that possess TMR could use the same mechanism to insert their genome into telomeres of host chromosomes.

A second model for HHV-6 integration is based on the putative integrase HHV-6 U94. U94 encodes a 490 amino acid protein and is unique to HHV-6A and HHV-6B. It has homology (24% identity) to the Adeno-Associated parvovirus (AAV) Rep78/68, a non-structural protein that is essential for AAV integration into chromosomes 19 [34–36]. Besides the similarity, U94 contains the conserved domains of Rep78/68 including the DNA binding and endonuclease domain at the N-terminus as well as the helicase and ATPase domains at the C-terminus [22]. Considering that Rep78/68 is essential for AAV integration and that U94 expression can complement an AAV Rep78/68 deletion mutant [37] suggests that U94 may play a role in HHV-6 integration. U94 possesses single-stranded DNA binding activity [20,38,39] and interacts with the TATA-binding protein [39]. Recent studies indicate that U94 binds telomeric DNA sequences and behaves as a 3′–5′ exonuclease (Trempe and Flamand, unpublished data). A model for U94-dependent integration of HHV-6 into human telomeres is proposed in Figure 3b. Through its DNA-binding and exonuclease activities, U94 would interact with the D-loop structure and remove the protected invading chromosomal end. This would result in the disruption of the T-loop structure and the generation of a 3′ recessed chromosome end. Simultaneously, U94 would attack the HHV-6 genome from its extremities generating a 5′ overhang at the DR_R that is complementary to that of the chromosome. These strands would anneal and polymerases/ligases would fill and close the gaps. As described for the BIR integration, the pac1 at the DR_L end would be lost by erosion followed by telomeric extension using the viral TMR as template.

(Figure 3 Legend Continued) genome. The terminal pac1 sequence would be lost by erosion and the adjacent TMR could be used to restore telomere sequences at the end. This integration process could occur independently of viral proteins. **(b)** U94-dependent integration process. Through its 3′–5′ exonuclease activity, U94 could process the ends of the viral genome and the telomeric D-loop structure, causing the T-loop structure to unfold. This would generate compatible ends that could facilitate annealing of the virus genome and the host chromosome. Upon annealing, the strands would be completed by cellular polymerases/ligases. The terminal pac1 sequence would be lost by erosion and the adjacent TMR could be used to restore telomere sequences at the end.

Do the HHV-6 telomeric repeats facilitate integration into host telomeres? Are other herpesviruses that harbor TMRs able to integrate into the host genome?

As mentioned above, the HHV-6 genome harbors two TMR arrays within the DR regions: the perfect TMR at the right end and the imperfect TMR at the left end of the DR (Figure 1). The number of TMRs varies from 15 to 180 copies in clinical isolates [3,6,9,40]. It has been proposed that the TMR are involved in HHV-6 integration; however, no experimental evidence has been published yet. Deletion of the TMR in the HHV-6 genome resulted in a virus that replicates comparable to parental and revertant viruses (Wallaschek and Kaufer, unpublished data), indicating that the TMR are dispensable for lytic replication. Integration analyses of recombinant viruses that lack the TMRs are currently under investigation.

Besides Marek's disease virus (MDV) and HHV-6, a number of other herpesviruses harbor TMRs [41]. Among them are more than a dozen herpesviruses from the *Herpesviridae* and *Alloherpesviridae* family. These include members of the *alphaherpesvirinae* subfamily such as MDV, herpesvirus of turkeys and duck enteritis virus, the betaherpesviruses HHV-6A, HHV-6B and human herpesvirus 7 (HHV-7) as well as the gammaherpesvirus equine herpesvirus 2. Even the distantly related alloherpesviruses cyprinid herpesvirus 1–3 that infect various fish species including carp, gold fish and koi, harbor TMR at both ends of their genome. The conserved nature of the TMR in various herpesviruses suggests an important function of those repeat sequences. Integration into host telomeres was so far only shown for MDV, HHV-6A and HHV-6B [21••,23,42•], but it is likely that also other herpesvirus containing TMRs in their genome integrate their genetic material. It was recently shown that the viral TMRs can facilitate integration of the virus genome into host telomeres using MDV as a model for herpesvirus integration *in vitro* and *in vivo* [42•]. As HHV-6A and HHV-6B also integrate into telomeres it is very likely that the viral TMRs are also involved in this process as proposed for the two integration models above. The closely related HHV-7 has not been reported to integrate into host chromosomes so far. In contrast to HHV-6, HHV-7 has a very narrow tropism as it infects only CD4 expressing cells [43]. Since only few cells are latently infected with HHV-7, it is very difficult to identify these cells to determine the status of the virus genome within an individual. Intriguingly, HHV-7 does not encode a homologue of U94, suggesting that this protein might be the decisive factor for integration.

Another open question is whether germ line integration also occurs with herpesviruses other than HHV-6A and HHV-6B. In case of HHV-7, the virus likely does not infect gametes as they do not express CD4, providing a possible explanation why germ line integration was not observed for this herpesvirus so far. A recent report demonstrated that tarsier monkeys carry an endogenous herpesvirus closely related to HHV-6 in their genome termed Tarsius syrichta roseolovirus 1 [44]. Unlike HHV-6, the genome of the tarsier monkey virus contains several mutations raising doubts that functional viruses could reactivate from the integrated state. Future studies should address if other herpesviruses that harbor telomeres are also able to integrate into the germ line.

Inherited chromosomally integrated HHV-6: major issues and top research priorities

Undoubtedly, the ultimate question is whether iciHHV-6 represents a risk factor in disease development. It is now well established that the self-renewal potential of cells is directly linked to telomere length and telomerase activity [45,46]. It is also known that the shortest telomere, not average telomere length, is critical for cell viability and chromosome stability [47]. Recent work by Huang et al. indicates that chromosomes carrying integrated HHV-6 often have the shortest telomeres [31••]. Once the number of telomeric repeated sequence (TMR) is reduced to 13, chromosomal instability is observed [48]. Several diseases are linked to telomere dysfunctions and/or telomerase mutations such as hematopoietic dysfunction, pulmonary fibrosis, liver disease, degenerative diseases and cancer [49–59]. Alterations within telomeric regions are therefore a likely cause for cellular dysfunctions linked to diseases. Intriguingly, Pellett et al. reported that iciHHV-6 is 2.3× more frequent ($P < 0.001$) in diseased (various diseases) individuals relative to healthy ones [60]. One potential caveat of this study is that the data was pooled from several small independent studies. The fact that the prevalence of iciHHV6+ individual varies between 0.2% and 2.9% depending on the geographical regions and population sampled (healthy versus diseased) likely affected the outcome of the study. Sampling of a large cohort (n = 50 000 subjects) of individuals aged over 40, when the prevalence of disease is greater, and from a region where the population is relatively homogenous (to minimize confounding factors) would represent an almost ideal way to address the clinical aspects of iciHHV-6. Access to medical records is key for such analyses. By comparing the prevalence of specific diseases in iciHHV-6 versus non-iciHHV-6 individuals, risk factors could be estimated. Once identified, more precise questions pertaining to the mechanisms causing disease could be addressed. In addition, integration of the virus in non-iciHHV-6 patients during latency could also alter cellular function of target cells such as T cells, monocytes, neuroglial cells. This could also influence co-infections with other pathogens or increase the risk of autoimmune disease.

Another area worth investigating is the ability of HHV-6 to infect gametes. To facilitate incorporation into the germ line, HHV-6 must infect gametes and integrate its genome into a host chromosome. This integration most likely occurs in an ovum or sperm progenitor cells, thereby increasing the likelihood transferring the HHV-6 genome into an embryo. Intriguing work by the Hollsberg group indicates that HHV-6 is present in sperm of healthy males and that the virus can bind to sperm cells. This binding mechanism would allow transport of the virus along with the sperm cells to the ovum [61•]. Another possibility is that HHV-6 can reactivate from infected sperm cells and spread to a fertilized egg cell. Whether a haploid chromosome content influences integration remains unknown. Clearly more work on this subject is needed to fully appreciate the initial steps leading to iciHHV-6.

Lastly, the mechanism that allows mobilization of the integrated HHV-6 genome, resulting in reactivation and pathogenesis, remains a fundamental question. Recent studies *in vitro* [21••] and *in vivo* [63•] provided some evidence that integrated HHV-6 can indeed mobilize its genome and reactivate. Two reports suggest that viral excision could occur through the formation of T-circles [31••,62]. These T-circles would arise from recombination events between HHV6 DR regions and result in the generation of a full length circular viral genome containing a single DR. This genome would then serve as template for rolling-circle replication of the virus genome, resulting concatemeric viral DNA. Further convincing evidence was recently provided by Endo et al. that reported pathogenesis from reactivated iciHHV-6A in a Japanese infant with X-SCID [64••]. The profound immunosuppression observed in X-SCID was most likely a key-contributing factor for the observed uncontrolled viral replication. From these observations, two major research priorities emerge. First, in the absence of safe and highly effective anti-HHV-6 drugs, the development of immunotherapeutic approaches to prevent/control HHV-6 reactivation is warranted. HHV-6 specific T cells recognizing peptides derived from the U11, U54 and IE1 proteins have recently been identified [65–68]. Whether these could prevent HHV-6 reactivation and disease should be addressed in a clinical setting. The second research priority relates to the transplantation of organs derived from iciHHV-6+ individuals. Even though almost everyone is already infected with HHV-6, the burden of latently infected cells is likely minimal compared to the number of HHV-6-infected cells introduced upon transfer of organs such as kidneys, livers or even bone marrow cells from an iciHHV-6+ donor. Organs from iciHHV-6+ donors would represent an important reservoir of latently infected cells, possibly reactivating considerable amounts of HHV-6. In addition, cells of iciHHV-6 patients are known to express viral RNAs in the absence of reactivation. Cells expressing HHV-6 proteins would be attacked by the immune system, which could explain, at least in part, idiopathic chronic organ rejection. Realizing that the demand for organ exceeds organ donation, monitoring of iciHHV-6 status of organ donors should be determined prior transplantation to avoid adverse effects and ensure proper diagnosis and treatment by the clinicians.

Conclusion

Initially considered an oddity among virologists, HHV-6 integration is now considered a part of the natural HHV-6 life cycle. Establishment of latency by integration of the virus genome into host chromosomes allows HHV-6 to minimize detection by immune effector cells, ensuring its long-term persistence. It should be pointed out that HHV-6 integration has so far been only observed in iciHHV-6 individuals. The search for cells carrying integrated HHV-6 from a non-iciHHV-6 is still ongoing. Recent advances have started to unravel how these viruses might excise themselves from the integrated state. Although the processes leading to integration remain elusive for the most part, the medical consequences associated with iciHHV-6 are now starting to be recognized as a risk factor for disease development. Large-scale population studies and systemic monitoring of iciHHV-6 status would provide conclusive answers to the biological and medical consequences associated with iciHHV-6.

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