Herpesvirus Genome Integration into Telomeric Repeats of Host Cell Chromosomes

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Abstract

It is well known that numerous viruses integrate their genetic material into host cell chromosomes. Human herpesvirus 6 (HHV-6) and oncogenic Marek's disease virus (MDV) have been shown to integrate their genomes into host telomeres of latently infected cells. This is unusual for herpesviruses as most maintain their genomes as circular episomes during the quiescent stage of infection. The genomic DNA of HHV-6, MDV, and several other herpesviruses harbors telomeric repeats (TMRs) that are identical to host telomere sequences (TTAGGG). At least in the case of MDV, viral TMRs facilitate integration into host telomeres. Integration of HHV-6 occurs not only in lymphocytes but also in the germline of some individuals, allowing vertical virus transmission. Although the molecular mechanism of telomere integration is poorly understood, the presence of TMRs in a number of herpesviruses suggests it is their default program for genome maintenance during latency and also allows efficient reactivation.

INTEGRATION OF VIRAL GENOMES INTO HOST CELL CHROMOSOMES

Virus integration: integration of the virus genome into host chromosomes

Integration of the genetic material of viruses into the host genome has been known for bacteriophages since the early 1950s. It began as a theory built on the fact that lysogeny, the bacteriophage life cycle during which no progeny viruses are produced, could be induced by environmental cues; seminal genetics experiments subsequently proved that temperate bacteriophages including λ, P1, and P2 incorporate their genetic material into the bacterial chromosome (1–5). The phages reactivate periodically under conditions favorable for replication, such as stress (e.g., following UV radiation or treatment with certain chemicals) and the presence of uninfected host cells. Otherwise, they remain in the dormant integrated state. It was recognized immediately that such mobilization of viral sequences not only allows initiation of virus replication at the optimal time point but also serves as a tool for transduction of host genetic elements to new host cells (5).

Following these observations in bacteriophages, investigators hypothesized that animal viruses may also be able to integrate their DNA into the genomes of host cells (6); serological assays soon indicated that Shope rabbit papilloma virus had this ability (7). More than 20 years later, human papillomavirus (HPV) types 16 and 18 were shown to integrate into cell lines derived from cervical carcinomas; this constituted one of the most important findings linking some HPV types with cervical cancers (8, 9). In the closely related polyomaviruses, physical integration of viral into host DNA was revealed by elegant biochemical experiments that combined RNA-DNA hybridization with alkaline $CsCl_2$ gradient centrifugation (10, 11). The same methodology was applied to demonstrate adenovirus integration into the host genome (12). Unlike in the cases of polyomaviruses and papillomaviruses, however, the biological consequences of such integration and the oncogenic potential of adenoviruses in their natural hosts remain controversial.

Whereas the integration of the genomes of DNA viruses into host chromosomes may seem somewhat natural, the integration of parts or all of the genomes of RNA viruses was initially quite counterintuitive. It took landmark findings worth two Nobel Prizes to unravel the mechanisms of retrovirus RNA replication and the genomic integration of Rous sarcoma virus (RSV), a pathogen of birds. The discovery of reverse transcriptase provided an explanation for how proviral DNA could be generated from RNA virus genomes (13, 14). In the years following Temin's (13) and Baltimore's (14) independent experiments published in 1970, the Varmus/Bishop and Vogt laboratories demonstrated the integration of RSV sequences in permissive and nonpermissive host cells (15). The authors made use of an improved technique based on that used by Sambrook and coworkers (10) a few years earlier for the polyomavirus simian virus 40 (SV40) to provide "direct physicochemical" evidence for the sequence of events that results in retrovirus genome integration, replication, and cellular transformation.

Whereas the mechanisms and biological consequences of integration into host genomes are well established for many different species of the retrovirus family, the integration of flaviviral and bornaviral sequences into host genomes remains a conundrum. In the flavivirus case, genomic integration of a DNA copy that represents a portion of the positive-sense RNA genome was demonstrated for both the vertebrate and invertebrate host. This finding may provide an explanation for the persistence described for some flavivirus species (16, 17). Finally, whole-genome sequencing for mammals of different orders revealed the presence of genomic DNA identical to the sequences of bornaviruses, agents with negative-sense RNA genomes (18, 19). The biological or even pathological consequences of such integration events remain to be determined for both of these RNA viruses. Similarly, the mechanisms of integration are hard to fathom, especially for flaviviruses that exclusively replicate in the cytoplasm. An overview of viruses that integrate their genetic material into host chromosomes is provided in **Table 1**.

Table 1 Viruses that integrate their genetic material into host DNA

Virus	Evidence	Mechanism	Reference(s)
RNA viruses			
Retroviruses	Presence of viral sequences in the host DNA and of cellular DNA in the virus	Reverse transcription and provirus integration	15, 135
Bornaviruses	Sequencing of the genomes of humans and other mammals	Endogenous retrotransposon (LINE-1)?	18, 19
Flaviviruses	Hybridization and sequence analysis of mosquito vectors	Endogenous retrotransposon?	16, 17
DNA viruses			
Bacteriophages	Genetic segregation and inheritance of temperate phages	Integrase	1, 3, 4, 136, 137
Adeno-associated viruses (parvoviruses)	Southern blot analysis	Nicking activity of viral Rep proteins	138, 139
Adenoviruses	Labeling of viral DNA with ^3H and $CsCl_2$ density centrifugation of infected-cell DNA	Unknown	12
Papillomaviruses	Demonstration of integrated DNA in carcinomas and cell lines	Double-strand breaks of host DNA and "random" integration	7, 8
Polyomaviruses	Hybridization and gradient centrifugation (simian virus 40)	Unknown	10, 11
Herpesviruses	Fluorescence in situ hybridization, germline transmission	Integration in telomeres via telomeric repeats present in the virus	36

HERPESVIRUS GENOME MAINTENANCE DURING LATENCY

The hallmark of replication for all known herpesviruses is their bipolar life cycle, which distinguishes productive, lytic replication from a state of quiescence termed latency. Latency is characterized by the absence of the production of infectious virions but the continued presence of the viral genome in infected target cells. Replication of the viral genome during the lytic phase involves at least seven virus-encoded proteins that are highly conserved among members of the Herpesvirales order. These proteins are produced in the early phase of lytic replication and provide the enzymatic machinery for efficient replication of viral DNA that is then packaged in preformed nucleocapsids (20). Production of viral DNA in lytically infected cells was always hypothesized to involve rolling circle replication following the circularization of viral genomes immediately after single-unit viral genomes are injected into the nucleus. The release of viral DNA from the incoming nucleocapsid at nuclear pores was shown for a number of members of the Herpesviridae family (20–22). However, at least in the case of the prototype alphaherpesvirus herpes simplex virus 1 (HSV-1), also known as human herpesvirus 1 (HHV-1), circularization was not observed in lytically infected cells. Rather, high-molecular-weight DNA was observed by Gardella gel analysis

Herpesvirus: large enveloped DNA virus

Latency: phase in the life cycle of some viruses when no infectious virions are produced but the viral genome remains present in infected cells

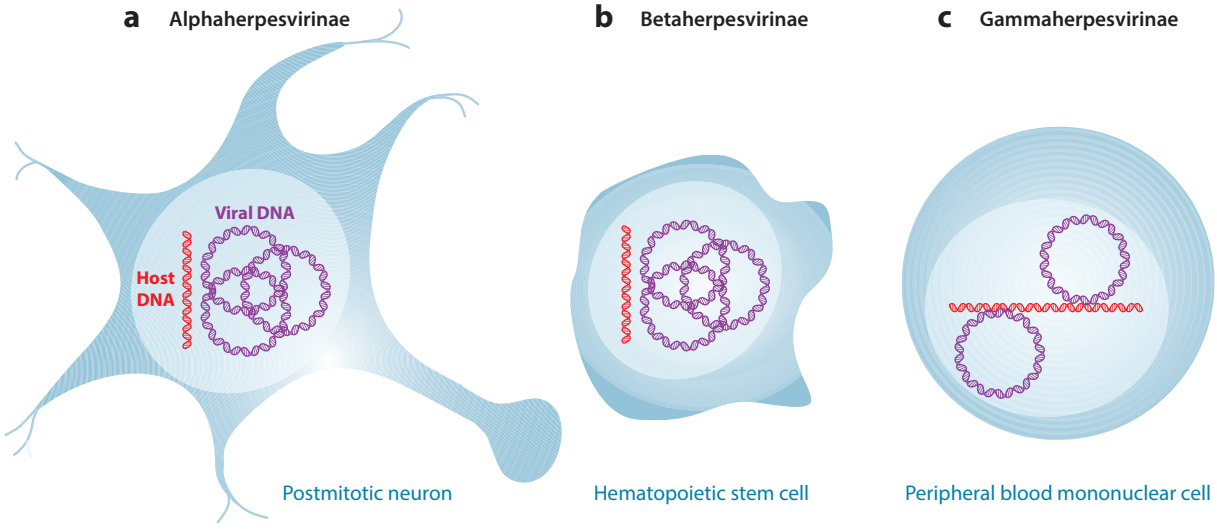

Figure 1

States of viral DNA during herpesvirus latency. Shown are the genomic states of viral DNA for members of the Herpesviridae subfamilies and its physical association with the host genome. (*a*) In the case of neurotropic Alphaherpesvirinae, latency is established in irreversibly postmitotic (terminally differentiated) neurons. Latent viral DNA is present in the nucleus in the form of circular episomes that do not seem to be physically associated with host chromosomes. (*b*) Members of the Betaherpesvirinae establish latency in differentiating cells such as hematopoietic stem cells. Viral DNA is present as episomes in multiple copies, but the association with the host chromosome is largely unknown. (*c*) Members of the Gammaherpesvirinae, most notably Epstein–Barr virus, establish latency in rapidly dividing peripheral blood mononuclear cells. Replication of viral episomes is tightly linked to that of host DNA during cell division. The latent (plasmid) origin of replication (oriP) and EBNA-1 (Epstein–Barr nuclear antigen 1) regulate the process. Viral episomes are tightly associated with the host chromosomes through tethering to telomeres via TRF-2 (telomerase repeat binding factor 2).

of viral DNA at various time points after infection. Jackson and DeLuca's (23) seminal experiments yielded no evidence of circular replication intermediates, even under conditions that would prevent late protein expression in cells lytically infected with wild-type HSV. In contrast, circular intermediates were readily observed when the cells were infected with a mutant virus that fails to express the viral immediate-early ICP0 protein and persists in a quiescent state in infected cells (23).

Whereas the establishment and maintenance of alphaherpesvirus genomes in latently infected cells have been well studied and documented (**Figure 1**), the nature of latent genomes of the prototype betaherpesvirus, human cytomegalovirus (HCMV; also known as HHV-5), is not well understood. There are a number of reports on the epigenetic regulation of viral transcription in latently infected cells, particularly in $CD34^+$ hematopoietic stem cells (reviewed in 24), yet the physical state of the HCMV genome in those cells is less clear. Although circular (episomal) DNA seems to be present in $CD14^+$ cells in the peripheral blood of infected individuals (**Figure 1**) (25), it remains unclear how viral episomes would be replicated and maintained in rapidly dividing bone marrow cells (26). The nature of the latent viral genome of the betaherpesvirus human herpesvirus 6 (HHV-6)—which was recently separated into two distinct species, HHV-6A and HHV6-B (27)—seems to be very different from that of HCMV, because virus genome maintenance is probably ensured by integration into the host genome, as we discuss in more detail below.

HHV-6: human herpesvirus 6

Virus genome maintenance: preservation of the virus genome in infected cells

In the case of Epstein–Barr virus (EBV; also known as HHV-4) and Kaposi sarcoma–associated herpesvirus (KSHV; also known as HHV-8), both members of the *Gammaherpesvirinae*, countless

studies have shown that the viral genomes in latently infected B and T lymphocytes are episomal in nature (**Figure 1**). Viral DNA enters the episomal state after nuclear delivery of incoming viral DNA, chromatinization, and epigenetic modification. These processes seem to be regulated mainly by viral tegument proteins that usurp DNA-damage responses and disrupt promyelocytic leukemia nuclear bodies (reviewed in 28). The episomes are replicated by the interaction of the viral proteins EBNA-1 (Epstein–Barr nuclear antigen 1, in EBV) and LANA (latency-associated nuclear antigen, in KSHV) with the plasmid origin of replication (29, 30). Tethering of the episomes to host telomeres, primarily via TRF-2 (telomeric repeat binding factor 2) (28, 31, 32), allows faithful segregation of replicated episomes to daughter cells during mitosis. Although integration of EBV DNA in some types of cancer, such as nasopharyngeal carcinoma, has been reported, it remains unclear whether the genomic structures identified in these reports represent true integration events or would also be consistent with tight telomeric association of latent viral DNA.

Telomeres: protein-associated repeat sequences that protect the ends of chromosomes from shortening and damage

TMR: telomeric repeat [$(TTAGGG)_n$]

MDV: Marek's disease virus

HHV-7: human herpesvirus 7

Perfect TMRs: TMR arrays containing 10 to 100 perfect hexameric repeats

Imperfect TMRs: TMRs disrupted by similar or unrelated repetitive sequences

HERPESVIRUS TELOMERIC REPEATS

Telomeres are specialized structures at the distal ends of eukaryotic chromosomes. In vertebrates, telomere sequences are highly conserved and comprise thousands of copies of the hexanucleotide TTAGGG associated with numerous proteins. Together, they form a very stable ribonucleoprotein complex that protects chromosome ends. A number of herpesviruses harbor arrays of telomeric repeats (TMRs), identical to those of the host, in their genomes. The presence of TMRs in a herpesvirus genome was first described for HHV-6 and Marek's disease virus (MDV) in 1988 (33). A few years later, TMRs were also detected in human herpesvirus 7 (HHV-7) (34). With the onset of high-throughput sequencing, many herpesvirus genomes were completely sequenced. Analysis of these genomes revealed that besides MDV, HHV-6, and HHV-7, several other members of Herpesviridae and Alloherpesviridae, the two families within the order Herpesvirales, also harbor arrays of TMRs (**Table 2**). Species from all three subfamilies of the Herpesviridae, Alpha-, Beta- and Gammaherpesvirinae, contain TMRs, although they are genetically quite diverse and specify class A, C, D, or E genomes (**Table 2**). Class A genomes consist of a unique sequence (U) that is flanked by a direct repeat region (DR). Class C genomes also harbor terminal sequences that contain a variable number of tandem repeats, as well as an internal set of direct repeats that is unrelated to the terminal sequences. Class D and E genomes contain two unique regions (U_L and U_S) that are each flanked by terminal and internal inverted repeats (TR_L/IR_L and TR_S/IR_S). The TR_L/IR_L are very short in class D genomes, whereas they are often more than 10 kbp long in viruses with class E genomes. Most herpesviruses with TMRs have class A or E genomes and, therefore, harbor them at their genome termini. In class A genomes, the TMRs are located at both ends of the DR sequences, which ensures the localization of TMRs at either end of the linear genome (**Figure 2**). The only exception is equine herpesvirus 2 (EHV-2), in which the TMRs are present in the center of the DR regions. The only herpesvirus with a class C genome harboring TMRs is ovine herpesvirus 2 (OvHV-2), in which the TMRs are found within the last repeat region (R10) at the end of the genome (**Figure 2**). In class D genomes, the repeats are at the terminal ends of the inverted repeat short (R_S) sequences flanking the U_S region. Similarly, class E genomes harbor TMRs at the junctions between the R_S sequences and the inverted repeat long (R_L) sequences, regions also known as the *a*-like sequences. The *a*-like sequences are present at the internal and terminal repeat junctions and are often duplicated during DNA replication (35).

The TMR arrays in herpesvirus genomes vary to some degree and can be classified into three different types. Perfect TMRs are composed of series of 10 to more than 100 perfect copies of the hexameric repeat TTAGGG. Imperfect TMRs are tandem arrays disrupted either by repetitive

Table 2 Members of the order Herpesvirales harboring telomeric repeat sequences in their genomes

Species	Genome type	Telomere integration	NCBI reference number
Herpesviridae			
Alphaherpesvirinae			
Marek's disease virus (MDV) [gallid herpesvirus 2 (GaHV-2)]	E	Yes (36)	NC_002229
Gallid herpesvirus 3 (GaHV-3)	E	Unknown	NC_002577
Herpesvirus of turkeys (HVT) [meleagrid herpesvirus 1 (MeHV-1)]	E	Unknown	NC_002641
Duck enteritis virus (DEV) [anatid herpesvirus 1 (AHV-1)]	D	Unknown	NC_013036
Marmoset herpesvirus (MarHV) [saimiriine herpesvirus 1 (SaHV-1)]	D	Unknown	NC_014567
Betaherpesvirinae			
Human herpesvirus 6A (HHV-6A)	A	Yes (94)	NC_001664
Human herpesvirus 6B (HHV-6B)	A	Yes (95)	NC_000898
Human herpesvirus 7 (HHV-7)	A	Unknown	NC_001716
Gammaherpesvirinae			
Equine herpesvirus 2 (EHV-2)	A	Unknown	NC_001650
Ovine herpesvirus 2 (OvHV-2)	C	Unknown	NC_007646
Alloherpesviridae			
Cyprinid herpesvirus 1 (CyHV-1)	A	Unknown	NC_019491
Cyprinid herpesvirus 2 (CyHV-2)	A	Unknown	NC_019495
Cyprinid herpesvirus 3 (CyHV-3)	A	Unknown	NC_009127

sequences very similar to the telomeric repeats or by completely unrelated sequences. Spaced TMRs contain patches of usually two or three repeats that are divided by longer spacer sequences of more than 18 bp. In several genomes, more than one type of TMR array can be found. The number of TMRs is often variable among different strains and isolates, suggesting that there is some flexibility. In almost all herpesvirus genomes, TMRs are located in close proximity to the predicted cleavage and packaging signals *pac1* and *pac2* (36, 37), which ensures their presence at the ends of the linear genomes. Intriguingly, most of the viruses that harbor TMR arrays establish latency in lymphocytes, which requires faithful replication of the viral genome together with cellular DNA during proliferation of latently infected host cells (38, 39). Several functions were proposed for viral TMRs. For example, they could allow the cell to recognize the viral genome as a mini-chromosome, which would allow evasion from the DNA-damage response that is initiated to free DNA ends. Alternatively, TMRs were proposed to play a role in the cleavage and packaging of

Spaced TMRs: TMR arrays containing short patches of repeats separated by longer spacer sequences

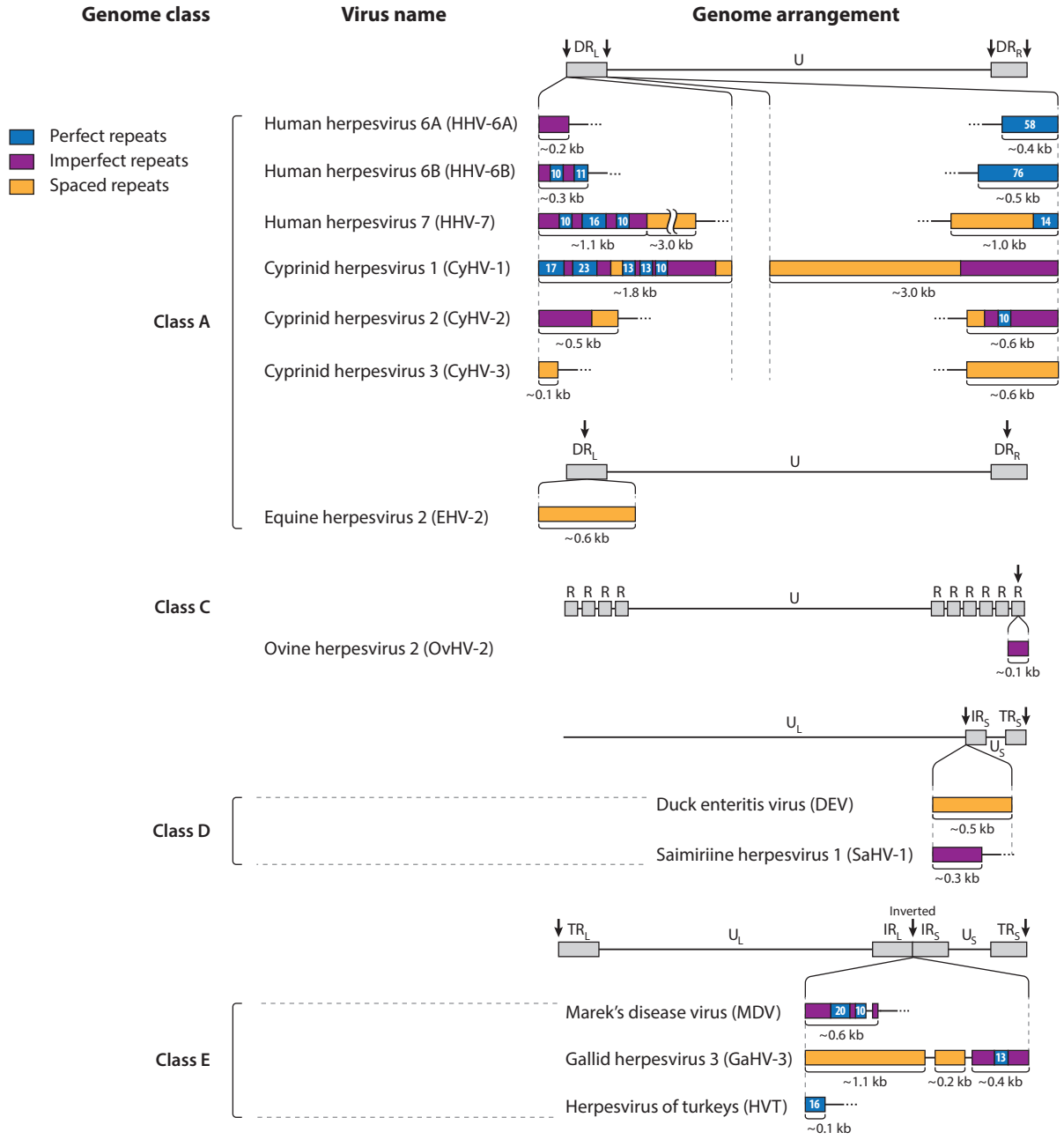

Figure 2

Overview of herpesvirus genomes that harbor telomeric repeat (TMR) arrays. The genome structures of the indicated viruses are shown based on their reference sequences present in NCBI (**Table 2**). The regions containing TMRs are highlighted with arrows. A focus on the regions harboring TMRs is shown with the three TMR classes: perfect (*blue*), imperfect (*purple*), and spaced (*gold*) TMR sequences. The numbers within the boxes indicate the quantities of perfect TTAGGG repeats. Abbreviations: DR_L, direct repeat left; DR_R, direct repeat right; IR_L, internal repeat long; IR_S, internal repeat short; R, repeat; TR_L, terminal repeat long; TR_S, terminal repeat short; U, unique; U_L, unique long; U_S, unique short.

viral DNA or in the integration of the virus genome into host chromosomes via homologous recombination. The latter mechanism would allow faithful replication of the genetic material of the virus during host cell proliferation.

Lymphoma: cancer of B or T lymphocytes, usually forming a solid mass

pUL12: protein encoded by the UL12 gene present in the unique long (U_L) region of alphaherpesviruses

ICP8: infected cell protein 8

MAREK'S DISEASE VIRUS

MDV [also known as gallid herpesvirus 2 (GaHV-2)], is a lymphotropic alphaherpesvirus that causes neurological disorders, immune suppression, and, primarily, malignant CD4 T cell lymphomas in chickens (40). Tumor cells derived from MDV-infected animals harbor the integrated viral genome in one or multiple chromosomes. Fluorescence in situ hybridization (FISH) analyses revealed that MDV genomic integration occurs predominantly at chromosome ends (41). Recently, pulsed-field gel electrophoresis experiments confirmed that MDV indeed integrates into, or at least in very close proximity to, host telomeres, but it has remained unclear whether the genome is located at the proximal (subtelomeric) or distal end of the telomeres (36). Investigations are currently ongoing to determine the exact integration site with next-generation sequencing.

The double-stranded MDV DNA genome is 180 kbp in size and consists of two unique sequences (U_L and U_S) flanked by terminal (TR_L and TR_S) and internal (IR_L and IR_S) inverted repeats (**Figure 2**) (42). TMRs were identified in all sequenced MDV genomes and are present in the *a*-like sequences, which are located at both ends of the linear genome as well as in an inverted orientation at the IR_L-IR_S junction. Upon circularization, one terminal copy of the *a*-like sequence is deleted (43). Within the *a*-like sequences, two TMR arrays are present. The first is a stretch of what we call multiple telomeric repeats (mTMRs), which specify variable lengths in different MDV strains (44); the second are the short telomeric repeats (sTMRs), which always consist of six copies of the TTAGGG repeat.

Systematic mutational analysis of the TMRs in the MDV genome revealed that neither mutation nor deletion of the mTMRs has an effect on MDV replication in vitro (36). However, MDV pathogenesis and tumor formation are severely impaired in the absence of the mTMRs. Only a few animals infected with TMR mutant viruses developed tumors (36). Analysis of tumor cells revealed that the integration efficiency of the mutants was highly reduced, as integration only occurred in a single locus that was not located in the host telomeres. This finding suggested that the directed integration mechanism was abrogated by mutation or absence of the viral TMRs (**Figure 3**). The observation that integration occurred in all tumor cells investigated led us to conclude that integration into the host genome is not simply a consequence of virus replication but rather a critical prerequisite for latent MDV infection that ensures faithful MDV genome maintenance in host cells during cell division.

MDV integration, like most recombination events, is likely dependent on proteins that facilitate the recombination between homologous sequences. MDV encodes a putative recombinase complex that consists of pUL12 and the HSV ICP8 ortholog encoded by UL29. The two putative complex partners are conserved among all herpesviruses, and their functions are well characterized in HSV. The complex resembles well-known recombination systems (45, 46) in which pUL12 acts as a 5′→3′ exonuclease that preferentially digests double-stranded DNA and generates 3′ single-stranded DNA overhangs (47, 48). ICP8 is a single-stranded DNA–binding protein (ssBP) that tightly binds and resolves secondary structures, resulting in protein-DNA filaments that are held in an extended configuration (49). Upon binding, HSV ICP8 facilitates reannealing of single-stranded DNA to complementary nucleic acid, a characteristic of all known ssBPs (50). The pUL12-ICP8 complex is thought to be involved in herpesvirus DNA replication, which requires a high degree of homologous recombination (51). The complex might also aid in resolving DNA branches that occur during DNA replication and would therefore allow unit-length genome

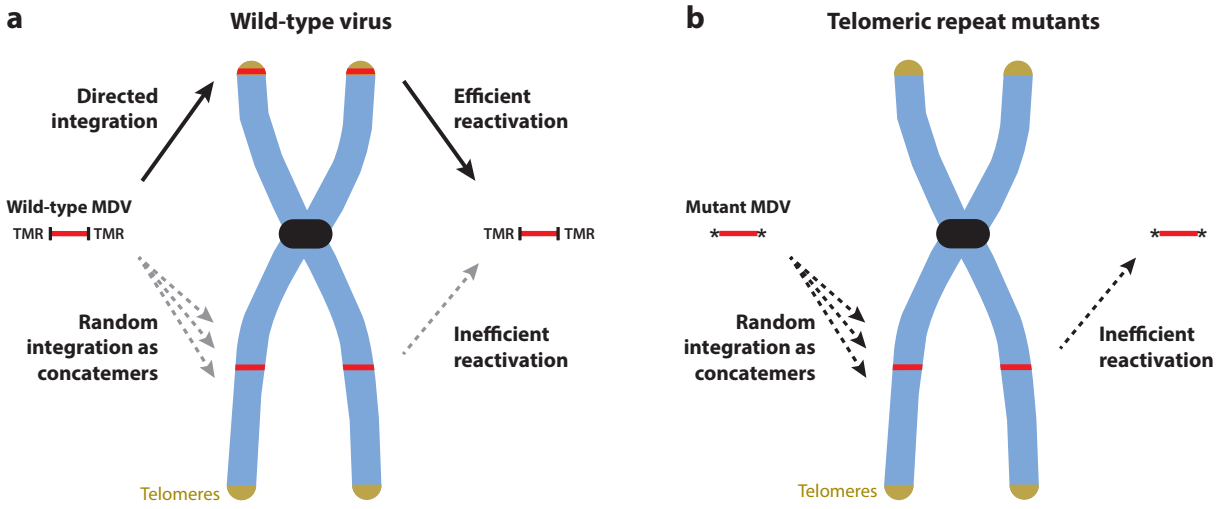

Figure 3

Model of herpesvirus (MDV) genome integration in the presence and absence of viral telomeric repeats. (*a*) In the case of wild-type virus, integration of viral DNA into host telomeres is efficiently mediated by telomeric repeats present in the viral genome. Targeted integration results in efficient tumorigenesis and reactivation from latency. Random integration events elsewhere in the chromosome are rare. (*b*) TMR-mutant viruses are unable to integrate into host telomeres, but integrate at random sites within chromosomes as concatemers. This random integration process is inefficient and reduces significantly both tumorigenicity and mobilization of virus genomes, which is needed for reactivation from the latent state of infection. Abbreviations: MDV, Marek's disease virus; TMR, telomeric repeat.

packaging into preformed capsids (45, 47). Because we hypothesize that MDV integration into host telomeres is facilitated by homologous recombination between virus and host telomeric repeats, we view it as likely that the virus-encoded putative recombinase complex is involved in the integration process. However, the role of pUL12 and ICP8 in integration of MDV and other herpesviruses needs to be formally addressed. In addition, cellular recombinases such as Rad51 and Dmc1 could also contribute to the process of integrating the viral genome into host telomeres (52).

Despite the essential role of MDV integration in lymphomagenesis, it is likely only the initial step in a process requiring viral proteins and noncoding RNAs that promote survival and proliferation of the host cell. The major oncogene of MDV encodes Meq, a basic leucine zipper (b-ZIP) transcription factor (53). Meq is a potent transcriptional activator, it complexes with the proto-oncoprotein c-Jun, it represses lytic viral gene products, and it modulates the cell cycle regulators p53 and RB (40, 54–56). Another factor important for MDV-induced tumorigenesis is viral telomerase RNA (vTR), a homolog of cellular telomerase RNA (TR). As part of the telomerase complex, vTR ensures increased telomerase activity at early stages of lymphomagenesis but also promotes tumor formation independently of its presence in the telomerase complex (57, 58). However, it is unclear whether vTR is involved in the integration process. In addition to Meq and vTR, several other factors are involved in lymphomagenesis, including the MDV chemokine vIL-8 (59, 60), MDV-encoded miRNAs (61, 62), and other gene products encoded in the IR_L of the MDV genome (63, 64). During infection in vivo, many cells become latently infected and initially transformed, but few of these cells ultimately survive the initial crisis and develop into lymphomas, as illustrated by the mono- or oligoclonal nature of lymphomas within one animal (41, 65). Tumor cells obtained from different organs of an animal have the same integration pattern, suggesting that integration occurs at a very early stage of the transformation

Reactivation: initiation of lytic virus replication from latently infected cells

process. In addition to the integration defect of MDV without TMRs, viral reactivation from tumor cells derived from animals infected with TMR mutants was significantly reduced, whereas parental and revertant virus species efficiently reactivated from corresponding tumor cells (36). This suggests that integration into telomeres might not only enable maintenance of the genome in latently infected (tumor) cells but also allow efficient genome mobilization (**Figure 3**).

U94: HHV-6-encoded protein that has homology to the adeno-associated virus type 2 rep replicase/integrase

Germline transmission: transmission of genetic information from parent to offspring via sperm or oocyte

Of note, two virus species in the genus *Mardivirus* that are closely related to yet distinct from MDV—gallid herpesvirus 3 (GaHV-3) and herpesvirus of turkeys [HVT; also known as meleagrid herpesvirus 1 (MeHV-1)]—also have type E genomes and possess TMRs in their *a*-like sequences (**Figure 2**). Despite the presence of the TMRs, neither GaHV-3 nor HVT induces lymphomas, likely because these viruses lack the critical oncogenes present in MDV. It is not known whether these two MDV relatives integrate their genomes into host telomeres during latency, but investigations are currently ongoing to address this question. Given the high homology in sequence and structure, we predict that GaHV-3 and HVT also integrate, because we contend that integration of viral genomes is "just" a means of faithful maintenance of viral genomes in dividing cells, whereas other viral factors are responsible for oncogenic transformation.

HUMAN HERPESVIRUS 6

In 1986, Salahuddin et al. (66) described this first member of the *Roseolovirus* genus, belonging to the Betaherpesvirinae subfamily, in patients with lymphoproliferative disorders. It was initially termed human B-lymphotropic virus (HBLV) but was later redefined as HHV-6. In the years following its discovery, various laboratories identified two genetically distinct variants, referred to as HHV-6A and HHV-6B, which display major differences with respect to their genetic, immunological, epidemiological, and biological characteristics (67). Therefore, based on the dissimilarities, HHV-6A and HHV-6B were finally classified as separate virus species by the International Committee on Taxonomy of Viruses in 2012 (27). Both viruses are ubiquitous, with seroprevalences of over 90% in the human population. Infection with HHV-6, supposedly transmitted through the saliva of infected individuals, occurs in early childhood before the age of 3 years (68, 69). Upon primary infection, mainly with HHV-6B, a febrile illness with a rash (known as roseola infantum, exanthema subitum, or sixth disease) ensues (70), which leads rarely to complications such as seizures and encephalitis (71, 72). HHV-6 (and HHV-7) can be considered opportunistic pathogens, which typically establish an asymptomatic, lifelong persistence in immunocompetent individuals but are linked to severe pathological conditions in the context of reactivation in immunosuppressed individuals. The reviews by Caselli and Di Luca (73) and De Bolle et al. (74) discuss the multitude of disease associations, many of which are the subject of extensive research and heated debate.

Activated $CD4^+$ T lymphocytes are the preferred cell type for efficient HHV-6 replication. Nevertheless, a variety of other cell types can be productively infected with HHV-6, which establishes latency most likely in cells of the monocyte and macrophage lineage. Although antigen and productive virus replication were not detectable, viral DNA was identified in those cells after more than 1 month in culture. Virus reactivation from the latent stage was achieved using either the phorbol ester trichostatin A or tetradecanoylphorbol acetate (75). HHV-6 can also latently infect early bone marrow progenitor cells (76), myeloid cell lines (77), an astrocytoma cell line (78), and an oligodendrocyte cell line (79), rendering them useful tools for in vitro experiments. In latently infected cells, four kinds of latency-associated transcripts of HHV-6B were determined (80), and expression of the U94-coding gene was shown to be significantly elevated (81).

The feature that sets HHV-6 apart from other human herpesviruses is its ability to integrate its genome into host chromosomes during latency (82–85) and thus to be vertically transmitted via the germline to subsequent generations (**Figure 4**). The condition known as chromosomally

integrated HHV-6 (ciHHV-6) is found in roughly 1% of the world's population, which makes research on this topic worthwhile (86–91). Individuals with ciHHV-6 carry the viral genome in every nucleated cell of the body (92, 93). Vertical transmission of ciHHV-6 was confirmed by demonstrating that parents and their offspring have identical sites of chromosomal integration and harbor the same virus strain (86, 87, 94–97). To date, 10 different integration sites of HHV-6 have been identified by FISH and all are in the telomeric region of chromosome 17p13.3, 18q23, 22q13.3, 9q34.3, 10q26.3, 11p15.5, 19q13.4, 1q44, or 18q2p (83–87, 94, 95, 98, 99). This poses the question of whether there are other integration sites yet to be discovered or whether there is a preference for specific chromosomes.

ciHHV-6: chromosomally integrated HHV-6

For reliable diagnosis of ciHHV-6, quantitative polymerase chain reaction using whole-blood DNA is commonly used. Once HHV-6 genome copies exceed 500,000 copies per mL of blood, a state of ciHHV-6 is considered highly likely. If unaware of the condition, clinicians might initiate antiviral treatment under the assumption of active HHV-6 infection, which carries substantial risks, especially for immunosuppressed patients (90, 92, 100, 101). Research in the near future will need to provide answers as to whether individuals with ciHHV-6 are predisposed to certain types of diseases, as proposed by Morissette & Flamand (102), or whether there is a serious danger that exposure to certain drugs and chemicals may reactivate the virus from its integrated state (103). Telomeres harboring ciHHV-6 were recently shown to be often among the shortest in somatic cells, making them susceptible to chromosomal damage. Telomere shortening increases the likelihood that cells undergo senescence or that telomere fusion events result in chromosomal instability and apoptosis (104).

Despite initial skepticism, the phenomenon of ciHHV-6 seems to have met widespread acceptance. Arbuckle et al. were the first to show, in 2010 (94) and 2013 (95), that HHV-6 is able to integrate into the telomeres of human chromosomes not only in vivo, which they demonstrated by the presence of ciHHV-6 in patient peripheral blood mononuclear cells (PBMCs), but also after in vitro infection of human cell lines such as JJHan and HEK293. Furthermore, no linear viral genomes or free circular episomes could be detected by Gardella gel analyses of latently infected cells. These findings raised the possibility that integration is not merely a whim of nature happening in some cell types such as germ cells; rather, it is a default mechanism by which HHV-6 establishes latency (**Figure 4**). Using specific primers for the virus genome and the subtelomeric region, Arbuckle et al. (94, 95) were able to demonstrate that integration occurs in the junction between chromosomes and telomeres. ciHHV-6 can also be reactivated from PBMCs from patients with ciHHV-6 and from cell lines harboring integrated HHV-6 produced in vitro. Finally, it was proven that the integrated HHV-6 genome can be mobilized from the chromosome (104), but the mechanism still is not well understood.

HHV-6A and HHV-6B have a class A genome that is 160 to 162 kbp long. It is composed of a 143–145-kb U region flanked by ~8-kb terminal DRs on the left (DR_L) and right (DR_R). A total of 119 open reading frames (ORFs) have been identified (105–107). HHV-6A and HHV-6B display an overall nucleotide sequence identity of 90% (106). Two viral factors encoded in the HHV-6 genome have been implicated in the integration of the viral genome into human telomeres: TMRs and the U94 protein (102). Two TMR arrays are present in the DR regions of the HHV-6 genome: perfect TMRs at the right genomic terminus and at the DR_L-U junction, and imperfect TMRs at the left genomic terminus and at the U-DR_R junction. The number of TMRs ranges from 15 to 180 copies in clinical isolates (33, 108–110). Based on the data on MDV, it is tempting to speculate that the HHV-6 genome integrates into host telomeres by homologous recombination of the viral TMRs with their host counterparts (36). The second viral factor likely involved in HHV-6 integration is the U94 protein, which is one of the most conserved proteins between HHV-6A and HHV-6B, differing by only 2.4% at the amino acid level. U94 is found only in HHV-6 (106, 111),

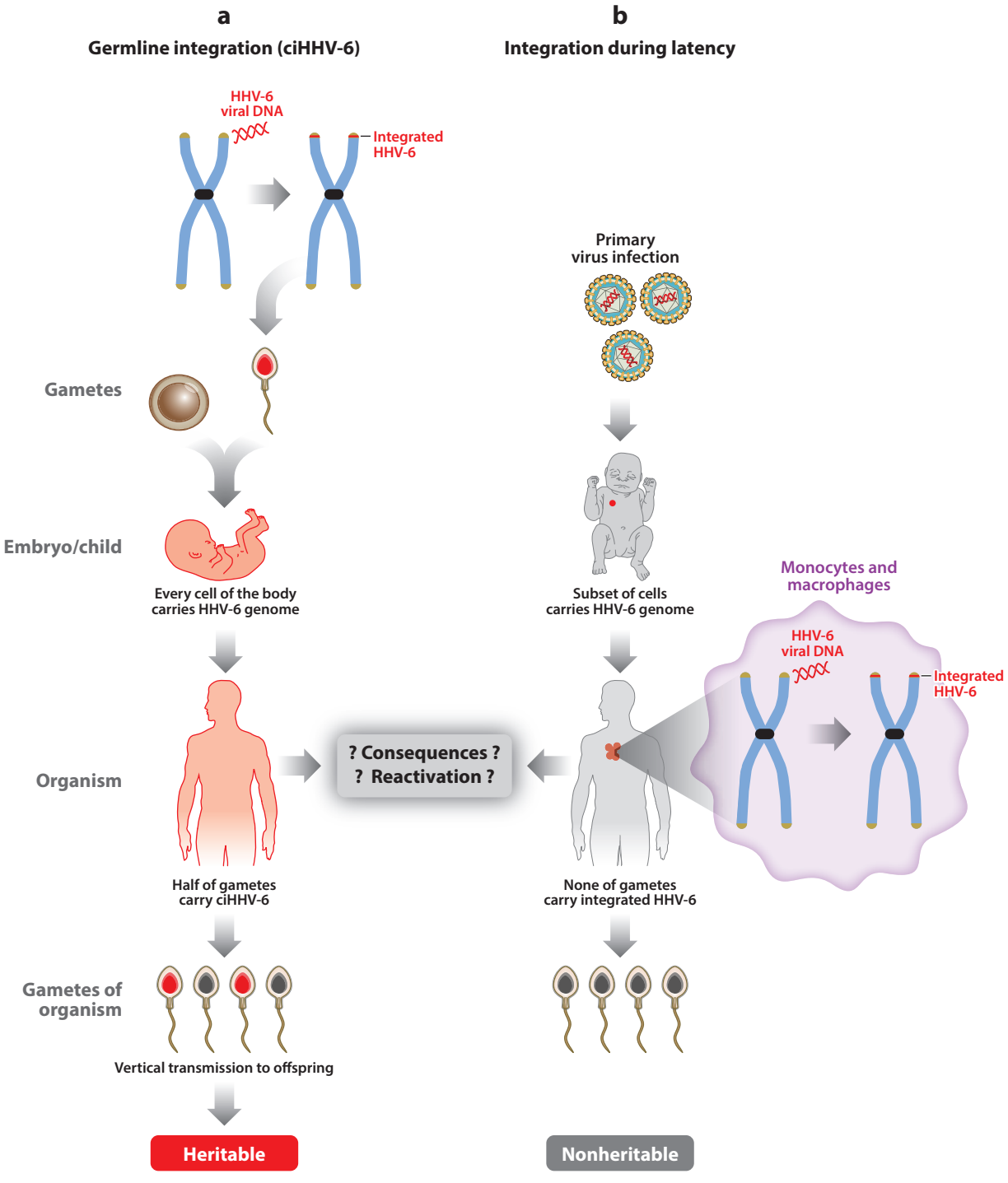
a
Germline integration (ciHHV-6)
b
Integration during latency
HHV-6
viral DNA
Integrated
HHV-6
Gametes
Embryo/child
Every cell of the body
carries HHV-6 genome
Organism
Half of gametes
carry ciHHV-6
Gametes of
organism
Vertical transmission to offspring
Heritable
Primary
virus infection
Subset of cells
carries HHV-6 genome
Monocytes and
macrophages
HHV-6
viral DNA
Integrated
HHV-6
? Consequences ?
? Reactivation ?
None of gametes
carry integrated HHV-6
Nonheritable

Figure 4

Integration of human herpesvirus 6 (HHV-6) in the germline and during latency. (*a*) Integration of HHV-6 into chromosomes in sperms or oocytes can result in an individual that harbors the virus in the germline. Chromosomally integrated HHV-6 (ciHHV-6) can subsequently be passed on to the offspring according to Mendelian inheritance laws. (*b*) Upon primary infection of an individual during childhood, the virus is also able to establish latency and integrate in cells of the monocyte and macrophage lineage. In contrast to germline integration, the virus is not vertically transmitted to offspring. The consequences of integration and the mechanism of genome mobilization during reactivation remain largely unknown.

but it exhibits a notable similarity (24%) to the human parvovirus adeno-associated virus 2 (AAV-2) Rep68 protein (112). Because HHV-6 can act as a helper virus for AAV-2 infection, it is possible that the virus pirated the *rep68* gene by nonhomologous recombination during coinfection with AAV-2 (113). AAV-2 Rep68 regulates viral gene expression and is essential for replication. It is a site- and strand-specific endonuclease with DNA-binding, helicase, and ATPase activity. Therefore, it is the driving force in site-specific integration of AAV-2 into chromosome 19q13.4 (114–120). Rep68-deficient AAV-2 no longer integrates into the host genome, or does so very inefficiently and randomly (119). The ability of U94 to complement replication of *rep68*-negative AAV-2 (121) strongly suggests that the HHV-6 and AAV-2 proteins have very similar biological functions. The similarity between the two proteins is underscored by the fact that the U94 C terminus corresponds to the helicase and ATPase domain of Rep68, whereas the N terminus resembles the Rep68 DNA-binding and endonuclease domain (112). It was reported that U94 has single stranded DNA–binding activity (122) and may operate at multiple levels by affecting the expression of virus replication proteins or acting directly on DNA replication (123, 124). Purified U94 has a nuclear localization and shows inhibitory activity on the replication of HHV-6 and other herpesviruses (125). Consistent with this inhibitory function, U94 is expressed only at low levels during lytic virus infection (111) but is found in latently infected PBMCs of healthy individuals in the absence of other transcripts. This suggests that U94 enables the establishment and/or maintenance of latent infection in lymphoid cells (81). In conclusion, it is quite likely that U94 functions as a potential integrase and recombinase that is instrumental for the integration process of HHV-6 during the establishment of latency.

HUMAN HERPESVIRUS 7

HHV-7, the third member of the *Roseolovirus* genus, was first isolated in 1990 by Frenkel and coworkers (126) from PBMCs of a healthy individual. Similar to HHV-6, HHV-7 is a ubiquitous agent, with seroprevalences in the human population of up to 90% (127). It is also acquired in early childhood by oral transmission, usually slightly later than HHV-6 (128–130). Disease associations are reviewed by Black & Pellett (131); they are similar to those of HHV-6 infections, but in most cases clinical presentations are milder.

The 145–153-kbp HHV-7 genome has the same class A structure as that of HHV-6. The U genome segment is approximately 133 kbp, and the DR_L and DR_R range from 6 to 10 kbp. Among the 84 to 86 ORFs, there is only one gene that is unique to HHV-7, which is termed U55B (132–134). The genomes of HHV-6 and HHV-7 are collinear across the entire genome. Overall, amino acid sequence identities between HHV-6 and HHV-7 core proteins vary between 41% and 75%. Intriguingly, HHV-7 does not encode a homolog of U94. HHV-7 also harbors TMR arrays that are very similar to those found in HHV-6, but the ones at the left end of the DR are slightly more complex and are variable between strains (73, 133, 134). It has not yet been shown that HHV-7 is able to integrate into human telomeres. Its similarity to HHV-6—especially

the presence of TMRs—would make integration similar to that observed for HHV-6 and MDV seem quite possible. At the same time, the absence of U94 might be the decisive factor, tipping the scale toward nonintegration. Future studies should, therefore, try to elucidate whether the TMR sequences of HHV-6 and HHV-7 are sufficient for integration and whether the U94 recombinase is essential in this process. If so, it would be tempting to analyze HHV-7 integration in the presence of U94 expression.

CONCLUSIONS AND DISCUSSION

After initial studies demonstrating the presence of temperate bacteriophage genomes in bacterial chromosomes, vertebrate retroviruses and DNA viruses, including adenoviruses, papillomaviruses, polyomaviruses, and herpesviruses, were shown to integrate their genetic material into the host genome. Although the biological consequences of such insertions remain unknown for some of these viruses, herpesvirus genome integration into host telomeres provides an elegant mechanism for the establishment of latent infection by ensuring genome maintenance in dividing host cells. Integration of herpesvirus DNA into host telomeres has been documented for MDV, HHV-6A, and HHV-6B, but the critical role of viral TMRs in the integration process has been proved only for MDV. However, the presence of various forms of TMRs in the genomes of numerous herpesviruses suggests that they serve a conserved and important function (**Table 2**). In this context, it needs to be confirmed whether integration is the default mechanism for genome maintenance during latency for herpesviruses with TMRs in their genomes. Similarly, the exact site of virus integration, whether intratelomeric or subtelomeric, should be addressed for each virus.

It has remained unclear whether integration of herpesviruses into telomeres requires viral and/or host DNA replication and whether viral genomes are integrated as single or multiple genome copies in the form of head-to-head or head-to-tail concatemers. The molecular mechanism of herpesvirus integration into host telomeres is poorly understood, although several proteins have been proposed to play roles in the process, including HHV-6 U94 and the putative recombinase complex pUL12/ICP8 encoded by all herpesviruses. Unfortunately, there is no experimental evidence for direct involvement of these proteins in the integration event, nor is there any evidence for cellular proteins modulating viral integration. We consider it likely that integration occurs only in a certain phase of the cell cycle, again posing the question of whether active host DNA replication is conducive to integration.

Even though vertical transmission of HHV-6 via chromosomal integration into the cells of the germline is well accepted, the consequences of ciHHV-6 are poorly characterized, and the possibility that other herpesviruses may integrate into the germline remains unexplored. The issue needs to be addressed, especially with respect to the epidemiology of infection. In summary, the integration of herpesvirus DNA into telomeres is a fascinating feature that is not a dead end for the virus, as initially assumed, but rather a way to faithfully maintain intact—and therefore potentially reactivating—virus genomes in rapidly dividing cells, allowing long-term virus persistence.

SUMMARY POINTS

1. A common feature of herpesviruses is the establishment of latency, during which the viral genome is maintained as a circular episome in the majority of viruses.
2. Integration of herpesviral DNA into the telomere region of host chromosomes has been shown for MDV, HHV-6A, and HHV-6B.

3. Vertical transmission of HHV-6 after chromosomal integration into the germline has been demonstrated.
4. Several herpesviruses harbor arrays of telomeric repeats identical to those in host telomere sequences (TTAGGG).
5. Viral telomeric repeats facilitate integration of the viral into the host genome.
6. Integration of MDV into the host genome is a prerequisite for lymphomagenesis.
7. Viral proteins that are likely involved in integration into host telomeres include U94 (HHV-6), pUL12 (MDV), and ICP8 (MDV).
8. Integration is not a dead end for the viruses, as integrated virus genomes are efficiently mobilized during reactivation.

FUTURE ISSUES

1. Do all herpesviruses that harbor telomeric repeats integrate their DNA into the host genome?
2. Is integration the default mechanism for genome maintenance during latency for herpesviruses with telomeric repeats in their genomes?
3. What are the exact sites of viral DNA integration in the telomeric region for different viruses?
4. Does integration occur in the form of a single viral genome or in multiple copies as concatemers?
5. The underlying mechanism of integration should be addressed, including the factors putatively involved in the process (HHV-6 U94, MDV pUL12/ICP8).
6. What are the pathological consequences of HHV-6 germline integration?
7. Is viral and/or host DNA replication required for integration?

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