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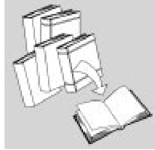
REVIEW



Recombination in alphaherpesviruses

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SUMMARY

Within the *Herpesviridae* family, *Alphaherpesvirinae* is an extensive subfamily which contains numerous mammalian and avian viruses. Given the low rate of herpesvirus nucleotide substitution, recombination can be seen as an essential evolutionary driving force although it is likely underestimated. Recombination in alphaherpesviruses is intimately linked to DNA replication. Both viral and cellular proteins participate in this recombination-dependent replication. The presence of inverted repeats in the alphaherpesvirus genomes allows segment inversion as a consequence of specific recombination between repeated sequences during DNA replication. High molecular weight intermediates of replication, called concatemers, are the site of early recombination events. The analysis of concatemers from cells coinfected by two distinguishable alphaherpesviruses provides an efficient tool to study recombination without the bias introduced by invisible or non-viable recombinants, and by dominance of a virus over recombinants.

Intraspecific recombination frequently occurs between strains of the same alphaherpesvirus species. Interspecific recombination depends on enough sequence similarity to enable recombination between distinct alphaherpesvirus species. The most important prerequisite for successful recombination is coinfection of the individual host by different virus strains or species. Consequently the following factors affecting the distribution of different viruses to shared target cells need to be considered: dose of inoculated virus, time interval between inoculation of the first and the second virus, distance between the marker mutations, genetic homology, virulence and latency. Recombination, by exchanging genomic segments, may modify the virulence of alphaherpesviruses. It must be carefully assessed for the biosafety of antiviral therapy, alphaherpesvirus-based vectors and live attenuated vaccines. Copyright © 2004 John Wiley & Sons, Ltd.

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INTRODUCTION

Herpesviruses evolved over a period of 400 million years [1,2] and *Alphaherpesvirinae*, an extensive subfamily which contains numerous mammalian and avian viruses (Table 1), separated from the other branches 180–210 million years ago [3]. The intranuclear replication of herpesviruses together with a sophisticated viral DNA replication machinery leads to an efficient proof-reading activity [4] and consequently a low rate of nucleotide substitution. The rate of synonymous substitution during the divergence of herpes simplex viruses 1 and 2 has been estimated to be 3×10^{-8} substitutions per site per year, a rate however at least 20 times higher than in mammalian genomes [5–7].

Despite this, numerous species of alphaherpesviruses have already been described, new ones

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Abbreviations used
BoHV-1, bovine herpesvirus 1; BoHV-2, bovine herpesvirus 2; BoHV-5, bovine herpesvirus 5; CpHV-1, caprine herpesvirus 1; CvHV-1, cervid herpesvirus 1; CvHV-2, cervid herpesvirus 2; DISC, disabled infectious single cycle; EHV-1, equine herpesvirus 1; FeHV-1, feline herpesvirus 1; GaHV-1, gallid herpesvirus 1; IBR, infectious bovine rhinotracheitis; PrV, pseudorabies virus; TK, thymidine kinase

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Table 1. The subfamily *Alphaherpesvirinae* is subdivided into four genus: *Simplexvirus*, *Varicellovirus*, *Mardivirus*, previously 'Marek's disease like viruses' and *Iltovirus*, previously 'Infectious laryngo-tracheitis-like viruses' [108]. Elk herpesvirus is genetically related to BoHV-1, and, although not officially classified, most likely belongs to this subfamily [8]. The group of simplexviruses related to HSV-1 and the group of varicelloviruses related to BoHV-1 are shown in bold

Genus	Species name	Alternative name	Abbreviation
Simplexvirus	**Human herpesvirus 1**	*Herpes simplex virus 1*	**HHV-1**
	Human herpesvirus 2	*Herpes simplex virus 2*	**HHV-2**
	Bovine herpesvirus 2	*Bovine mamillitis virus*	**BoHV-2**
	Cercopithecine herpesvirus 1	*B-virus*	**CeHV-1**
	Ateline herpesvirus 1	*Spider monkey herpesvirus*	**AtHV-1**
	Cercopithecine herpesvirus 2	*SA8*	**CeHV-2**
	Cercopithecine herpesvirus 16	*Herpesvirus papio 2*	**CeHV-16**
	Macropodid herpesvirus 1	*Parma wallaby herpesvirus*	**MaHV-1**
	Macropodid herpesvirus 2	*Dorcopsis wallaby herpesvirus*	**MaHV-2**
	Saimiriine herpesvirus 1	*Herpesvirus tamarinus*	**SaHV-1**
Varicellovirus	**Bovine herpesvirus 1**	*Infectious bovine rhinotracheitis virus*	**BoHV-1**
	Bovine herpesvirus 5	*Bovine encephalitis virus*	**BoHV-5**
	Bubaline herpesvirus 1	*Water buffalo herpesvirus*	**BuHV-1**
	Caprine herpesvirus 1	*Goat herpesvirus*	**CpHV-1**
	Cervid herpesvirus 1	*Red deer herpesvirus*	**CvHV-1**
	Cervid herpesvirus 2	*Reindeer herpesvirus*	**CvHV-2**
	Canid herpesvirus 1	*Canine herpesvirus*	**CaHV-1**
	Cercopithecine herpesvirus 9	*Simian varicella virus*	**CeHV-9**
	Equid herpesvirus 1	*Equine abortion virus*	**EHV-1**
	Equid herpesvirus 3	*Equine coital exanthema virus*	**EHV-3**
	Equid herpesvirus 4	*Equine rhinopneumonitis virus*	**EHV-4**
	Equid herpesvirus 8	*Asinine herpesvirus 3*	**EHV-6**
	Equid herpesvirus 9	*Gazelle herpesvirus*	**EHV-9**
	Felid herpesvirus 1	*Feline viral rhinotracheitis virus*	**FeHV-1**
	Human herpesvirus 3	*Varicella-zoster virus*	**HHV-3**
	Phocid herpesvirus 1	*Harbor seal herpesvirus*	**PhoHV-1**
	Suid herpesvirus 1	*Pseudorabies virus* (PrV)	**SuHV-1**
Mardivirus	Gallid herpesvirus 2	*Marek's disease herpesvirus 1*	**GaHV-2**
	Gallid herpesvirus 3	*Marek's disease herpesvirus 2*	**GaHV-3**
	Meleagrid herpesvirus 1	*Turkey herpesvirus 1*	**MeHV-1**
Iltovirus	Gallid herpesvirus 1	*Infectious laryngo-tracheitis virus*	**GaHV-1**

are still being discovered [8] and, even within a single herpesvirus species, virulence, biological and biochemical differences are the basis for further strain differentiation. Other mechanisms must therefore act together with mutations to contribute to herpesvirus evolution such as acquisition of cellular genes [9] and recombination.

Recombination encompasses four mechanisms: site-specific recombination, transposition, illegitimate and homologous recombinations; the latter two are described in the family *Herpesviridae* [10]. The mechanism of recombination is poorly understood in herpesviruses. It is, however, coupled with viral DNA replication and may require cell factors [11,12].

As early as 1955, the pioneer Peter Wildy identified HSV-1 recombinants from mixed inoculations between pairs of temperature-sensitive mutants [13]. The same use of temperature-sensitive mutants allowed derivation of recombinants within HSV-1

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[14] and between HSV-1 and HSV-2 [15]. Indeed, homologous recombination occurs at a very high rate under experimental conditions [16,17].

Given the low rate of herpesvirus nucleotide substitution, recombination can therefore be seen as an essential evolutionary driving force increasing the probability for a rare advantageous nonsynonymous mutation to spread, and later to be fixed, in the viral species. Although it seems relatively easy to study recombination in the laboratory, it is more difficult to investigate it in natural conditions. Therefore this phenomenon is likely underestimated. However, the biological properties of alphaherpesviruses act synergistically to favour recombination: high prevalence of infection in most mammalian and avian populations, muco-cutaneous sites of primary replication, high likelihood of coinfections, rapid lytic cycle, latent persistence interrupted by reactivation and virus replication at peripheral sites. Indeed a recent study demonstrates that a high recombination rate is observed in HSV-1 natural populations [18]. Latency of alphaherpesviruses provides an additional mechanism that helps to maintain a mutated or recombinant herpesviral genome over long periods in a small population.

Recombination could also be involved in the sudden emergence of new viruses combining a highly virulent phenotype with other characteristics. Recovery of virulence by recombination between two avirulent alphaherpesvirus strains has been demonstrated experimentally [19]. The presence of repeated sequences in herpesvirus genomes has led to the definition of six different classes, designated by the letters A to F [20]. Only two classes are found in *Alphaherpesvirinae*, namely class D in *Varicellovirus* genus and class E in *Simplexvirus* genus (Figure 1). The presence of inverted repeats inside the alphaherpesvirus genome has a fundamental importance. They have been shown to be involved in intramolecular recombination leading to nearly equal amounts of genomic isomers: two abundant isomers in varicellovirus genomes and four isomers in simplexvirus genomes (Figure 1). Nevertheless, recombination can occur at any site in the genome and not exclusively in these repeated sequences.

Homologous recombination is the most common mechanism encountered in alphaherpesviruses, although illegitimate recombination is also rarely described [21]. Homologous recombination requires closely related genomes, but for the creation of new virus variants, a certain level of sequence heterogeneity must be present in the genomes involved in recombination. In this respect, it is therefore extremely useful to study clusters of related alphaherpesviruses, such as the simplexviruses related to HSV-1 and the varicelloviruses related to bovine herpesvirus 1 (BoHV-1) (Table 1).

Class D genome

Class E genome

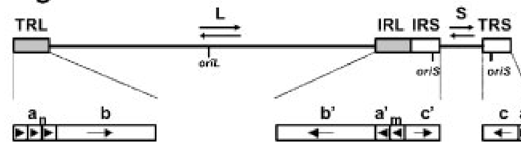

Figure 1. Organisation of *Varicellovirus* (class D) and *Simplexvirus* (class E) genomes. Class D genomes include two unique sequences, a long one (L) and a short one (S). The latter is flanked by two repeated and inverted sequences (internal repeat, IR; terminal repeat, TR). DNA replication generates equimolar amounts of two isomers which differ by the relative orientation of S and L segments (horizontal plain arrows). The L segment is predominantly observed in a single orientation, but low amounts of genomes with inverted L segments are observed in some varicelloviruses (horizontal hatched arrow). Replication of class E genomes generates equimolar amounts of four isomers due to the relative inversion of L and S segments (horizontal plain arrows). The positions of the origins of replication (ori_L and ori_S) are indicated. Repeated and inverted sequences are detailed: TRL (ab), IRL (b′a′), IRS (a′c′), TRS (ca). The left genomic terminus contains n repeats of sequence *a* followed by a single copy of sequence *b*. The right genomic terminus contains a unique copy of sequence *a* preceded by sequence *c*. Terminal sequences *ab* and *ca* are in an inverted orientation at the junction between unique segments L and S (b′a′m c′)

The goal of this paper is to review the importance of recombination not only as a phenomenon contributing to the evolution of alphaherpesviruses but also as an issue to be taken into account in the understanding of new outbreaks of alphaherpesviral diseases and for the biosafety assessment of resistance to antivirals, of live attenuated vaccines and of replicating or nonreplicating vectors.

RECOMBINATION AND DNA REPLICATION

Recombination between different strains of the same alphaherpesvirus can be studied at different levels of the lytic cycle, either by the characterisation of progeny viruses resulting from mixed inoculations or by the analysis of the concatemeric

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structures generated by the coinoculation of two distinguishable strains. Recombination in alphaherpesviruses is intimately linked to DNA replication [22,23]. Both viral and cellular proteins participate in this recombination-dependent replication [12,24]. DNA replication of alphaherpesviruses aims to produce from an infecting unit-length linear genome a high number of copies of identical viral genomes that will be packaged into newly formed capsids. Although extensively studied with HSV-1 as a model, alphaherpesvirus DNA replication remains a poorly understood mechanism. However, a model of HSV-1 DNA replication has been proposed [22,25] consisting of a cascade of events divided into three regulated steps that will be discussed separately.

Entry of the genome into the nucleus

The model suggests that HSV-1 DNA replication starts when the linear genome circularises in the host cell nucleus. This concept is based on cumulative observations reporting that, within hours after infection, end-joining of the HSV-1 linear genome ('endless' genome) has been found to occur [26,27]. This process is probably the result of end-to-end ligation of genomic termini or recombination between *a* sequences present in the inverted repeats flanking S and L genomic segments (Figure 1). Early genome circularisation was also observed for the alphaherpesviruses VZV [28] and pseudorabies virus (PrV) [29], and other herpesviruses such as human cytomegalovirus [30]. Circular genomes likely serve as a template for the second replication step which produces high molecular weight intermediates of replication (see below). The role of the circular genome as a prerequisite template for viral replication has been recently challenged by the use of Gardella gels which are able to separate linear from circular genomes [31,32]. With this technique, HSV-1 circular genomes were not detected in infected cells before the onset of DNA synthesis, leading to the surprising hypothesis that linear genomes could be the initial replication template rather than circular genomes (Figure 2).

DNA replication from the initial template and concatemeric DNA

The HSV-1 model of replication postulates that the circular genome serves as a template for a first round of theta replication starting from sequences known as origins of DNA replication [33,34]. There is no firm proof so far for the existence of theta replication intermediates. This bidirectional replication step would then switch, by an unknown mechanism, to a second step of monodirectional replication (using the rolling circle mode of replication) to produce high molecular weight intermediates of replication that are called concatemers (Figure 2). In concatemers, the termini of the newly formed viral genomes are fused in a head-to-tail arrangement [35]. This model of replication has also been challenged. Indeed, a complete digestion of concatemeric DNA with restriction enzymes that cut once per genome fails to render a large proportion of replicative intermediate DNA competent to migrate after pulsed field gel electrophoresis. Based on this observation, replicative DNA is not a long linear DNA molecule but contains frequent branches [36–42].

In HSV-1, recombination occurs within concatemeric DNA as early as concatemers can be detected [42]. Furthermore, recombination is an integral component of the DNA replication machinery, uses several cellular and viral factors, such as the HSV-1 single-stranded DNA-binding protein ICP8, but does not appear to require a specific enzyme that can be segregated from the pool of DNA replication factors [12,43]. Recombination is probably responsible for the branched structure of concatemers and is probably an inherent and important component of the DNA replication mechanism that significantly contributes to efficient synthesis of late concatemers, perhaps by recombinational deletion of circular genomes, generating additional templates for DNA synthesis [44] or by generating additional replication forks as each recombination event could act as a non-specific origin of replication. Recombination also plays a role with DNA replication to maintain genomic stability by repairing double-strand breaks [43].

The final step of the alphaherpesvirus DNA replication occurs when concatemers are cleaved at specific sites to form unit-length molecules that are packaged into newly formed capsids [45–48].

Recombination between genomic inverted repeats

Numerous reports concerning alphaherpesvirus recombination focused on segment inversion. In

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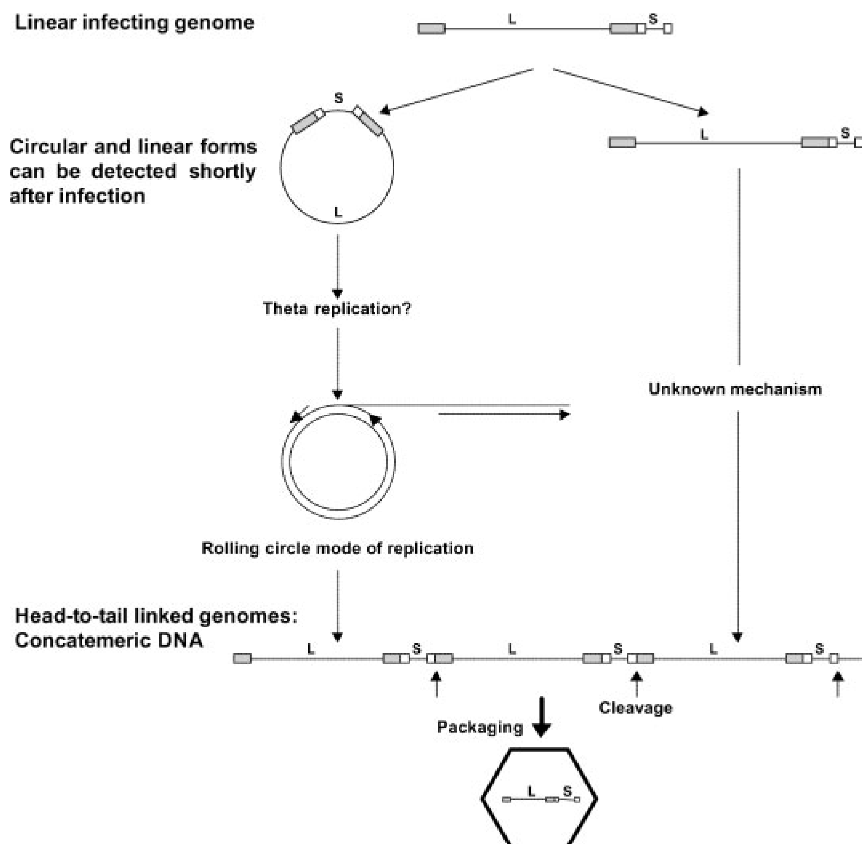

Figure 2. Model of replication and cleavage-packaging of alphaherpesvirus DNA. If the initial template is circular, replication is driven to a unidirectional replication following the rolling circle model. If the initial template is linear, replication must occur by another mechanism. Intermediates of replication consist of long concatemers made of genomic units in a head-to-tail arrangement. These concatemers are cleaved at specific sites and packaged in newly formed capsids

the case of HSV-1, a simplexvirus with a class E genome, it was postulated that genomic segment inversion is a consequence of specific recombination events between *a* sequences during the DNA replication process [27,49–53] (Figure 1). The cellular endonuclease G specifically cleaves HSV-1 *a* sequences and may be involved in the initiation of recombination at the *a* sequence leading to segment inversion [54]. However, any large set of inverted repeats that lies in a herpesvirus genome [53] or that is artificially inserted into the genome [44,55] is able to support genomic segment inversion.

In varicelloviruses BoHV-1 and equine herpesvirus 1 (EHV-1) with a class D genome, the L segment is predominantly fixed in only one orientation, called the prototype orientation, so that two isomers are detected in equimolar amounts in virion DNA due to the inversion of the S segment [20]. However, inversion of adjacent L segments also occurs and can be shown within concatemeric DNA for EHV-1 [56] and BoHV-1 [41]. Moreover, equimolar amounts of the four possible arrangements of adjacent L segment were identified within BoHV-1 concatemeric DNA, strongly indicating that recombination is an efficient mechanism linked to replication in alphaherpesviruses [41]. This result was surprising since starting from prototype genomes the rolling circle mode of replication is predicted to generate L segments in only one orientation within concatemeric DNA. Nevertheless an equimolar proportion of the four orientations is no longer present in the packaged DNA. As a consequence, the defect in the formation of genomes having the L segment in an inverted orientation appears to lie at the level of concatemer cleavage and packaging.

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The presence of inverted repeats provides therefore a convenient mechanism of genome isomerisation. However, their existence is not a prerequisite for recombination. This conclusion is supported by observations made in a betaherpesvirus, murine cytomegalovirus, where DNA replication is highly recombinogenic despite the fact that the genome contains no inverted repeats [44].

Study of recombination by characterisation of concatemeric DNA

Early recombination events between two strains of the same alphaherpesvirus can be studied at the concatemeric level [57,58]. The methodology requires the use of two strains distinguished by the presence of a unique restriction site within the genome of only one of the two strains [58]. After coinoculation of these two strains various forms of viral DNA can be detected following digestion with an appropriate endonuclease (Figure 3), e.g. unit-length genomes (due to the presence of the strain containing the unique restriction site) and higher-molecular-weight intermediates of replication (having for example, the length of two or three genomes) which indicate recombination events between genomes of the two strains within or between concatemeric DNA.

After cleavage and packaging, mixed concatemers will release parental as well as chimeric genomes (Figure 4). The methodology of mixed concatemers was used successfully in the study of recombination between BoHV-1 strains and between BoHV-1 and related ruminant alphaherpesviruses (see below) [57, Meurens *et al.*, unpublished results].

The study of recombination between viruses is frequently performed by using selected markers in the genome of parental viruses. Because multiple crossovers between selected markers can produce recombinant viruses still expressing the parental phenotypes, the question of 'invisible recombinants', i.e. true recombinants which are not detected in the experiment because they lack any selected marker, needs to be addressed. By escaping all the steps of concatemer cleavage, DNA packaging and egress of virions, the analysis of mixed concatemers avoids most of the bias introduced not only by invisible recombinants but also by non-viable recombinants and by dominance of a progeny virus over recombinants.

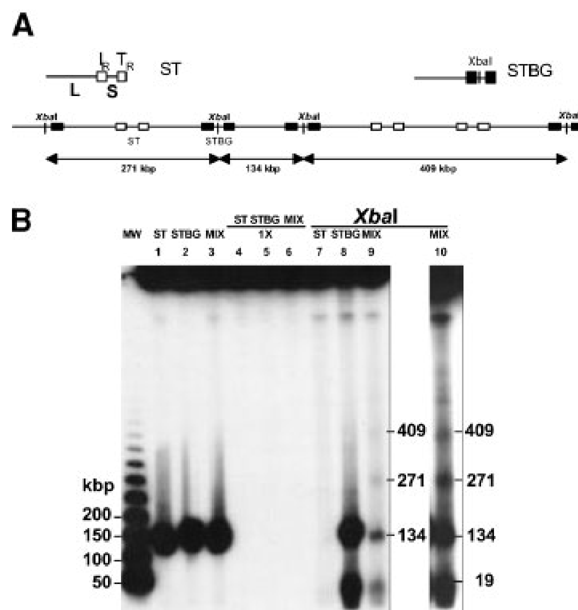

Figure 3. Mixed concatemeric DNA can be identified by pulsed field gel electrophoresis (PFGE). (A) Schematic representation of mixed concatemeric DNA and potential high molecular weight fragments generated after *Xba*I digestion. Inverted repeated sequences (IR and TR) derived from STBG and ST bovine herpesvirus 1 strains are represented by black and white boxes, respectively. (B) MDBK cells were either singly infected with ST or STBG or coinfected with both viruses. Plugs were prepared 30 h after infection and subjected to PFGE to produce concatemeric DNA free of unit-length genomes (lanes 1–3). Plugs that migrated once were then either mock digested (lanes 4–6) or digested with *Xba*I (lanes 7–9). Lane 10 is an overexposure of lane 9. (adapted from [57], with permission of American Society for Microbiology)

However, some recombination events will still be ignored even by concatemer analysis. Only full genome sequencing is able to overcome completely the problem raised by the invisible recombinants.

CLASSES OF RECOMBINANT VIRUSES

Intraspecific recombinants

Homologous recombination between strains of the same alphaherpesvirus species frequently occurs, both *in vitro* and *in vivo*. This result is not unexpected because the sequence homology is very high within a single herpesvirus species. This process has been described in several alphaherpesviruses: between simplexviruses, like strains of HSV-1 or HSV-2 [19,59,60], and between varicelloviruses, like strains of VZV, PrV, BoHV-1 or feline

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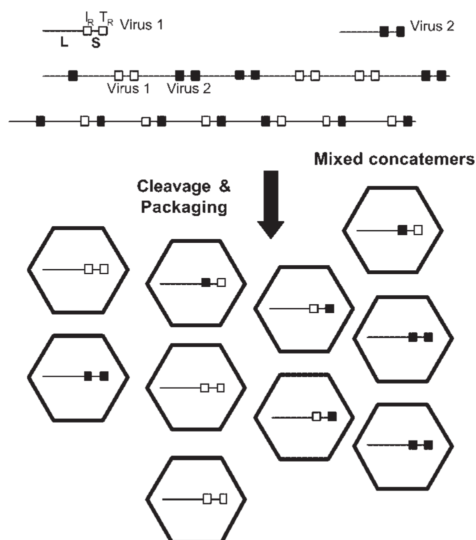

Figure 4. Schematic representation of mixed concatemeric DNA and potential genomes generated after cleavage. Black and white boxes represent inverted repeated sequences (IR and TR) from two different parental strains

herpesvirus 1 (FeHV-1) [17,61–63]. The rate of recombinant viruses is usually very high *in vitro*, e.g. 25% to 32% for BoHV-1 [57] and 10% to 21% for FeHV-1 [62]. However, *in vitro* studies do not reflect what happens *in vivo* and, moreover, some of the methods used to study recombination *in vivo* precluded the investigation of the following important features: (i) the evolution of the relative proportions of parental versus recombinant progeny populations during the course of the entire excretion in a single animal and (ii) the possibility of survival of recombinants after reactivation from latency. These questions were addressed in a recent experiment performed in cattle coinoculated, by the natural route of infection, with two BoHV-1 mutants [17]: (i) starting from two parental mutants, recombination generates a new progeny population composed of parental mutants and recombinant viruses at the same rate as *in vitro*; (ii) recombination is a frequent event *in vivo* since recombinants were detected in all coinoculated animals; (iii) the relative proportions of progeny populations evolve during the excretion period towards a situation in which two populations (including a recombinant virus) predominate largely without outcompeting the presence of the other two; and (iv) after reactivation from latency two viral populations including a recombinant virus were detected during the reexcretion period, providing evidence of a long lasting persistence of recombinant viruses in a population. These experimental findings are supported by the isolation in the field of intraspecific recombinants in different viral species: HSV-1 [18], VZV [64] and PrV [65].

Interspecific recombinants

Among alphaherpesviruses described in human and animals, most of them show too few sequence similarities to allow homologous recombination. Recombination was studied *in vitro* and *in vivo* between HSV-1 and HSV-2, which are closely related [15,66–70], and between HSV-1 and bovine herpesvirus 2 (BoHV-2) or HSV-1 and PrV [67]. Interspecific recombinant viruses were isolated between HSV-1 and HSV-2, while no recombination events were reported between HSV-1 and BoHV-2 or PrV [15,67,68,70]. More recently, recombination was assessed between ruminant alphaherpesviruses, a unique group of related alphaherpesviruses showing lower and higher sequence homologies than those observed between HSV-1/HSV-2 and HSV-1/BoHV-2, respectively (Table 1). Recombinants between identical or different strains of BoHV-1 were isolated at a frequency up to 30%, whereas only two recombinants between BoHV-1 and bovine herpesvirus 5 (BoHV-5) were isolated, and no recombinants between BoHV-1 and less closely related caprine herpesvirus 1 (CpHV-1) and cervid herpesvirus 2 (CvHV-2) were detected [16]. Despite a higher degree of nucleic acid homology (BoHV-1/BoHV-5: 82.3%, HSV-1/HSV-2: 75%), BoHV-5 did not recombine more with BoHV-1, than did HSV-1 with HSV-2 [16]. Interspecific recombination is detectable between alphaherpesviruses but the mechanism is poorly efficient when compared with intraspecific recombination [17,57,68]. Failure to detect recombinants between less closely related alphaherpesviruses does not exclude a recombination process as mixed concatemers between BoHV-1 and CvHV-2 and between cervid herpesvirus 1 and CvHV-2 have been identified in coinfected cells [Meurens, unpublished results]. Recombination between other closely related viruses, such as HSV-1 and B-virus or BoHV-1 and bubaline herpesvirus 1 or elk herpesvirus, needs also to be investigated. Interspecific recombinants rarely survive in natural conditions.

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Such recombinants have never been detected in nature [71].

FACTORS INFLUENCING RECOMBINATION

The most important prerequisite for successful recombination is coinfection of the individual host by different virus strains or species. Indeed, host coinfection might never occur between some viruses simply because their populations do not usually overlap in space and time. When this first step is reached, the different viruses must coinfect single cells to allow the recombination process. Consequently all the factors affecting the distribution of different viruses to shared target cells, and thereby limiting or increasing the likelihood of cellular coinfections need to be considered. On this basis, factors influencing recombination can have three sources: the host, the cell and the virus.

Among host factors, innate or adaptive immune responses and entry or inoculation site can be envisaged. Transmission of viruses between individuals of the same species or by crossing the species barrier requires either direct or indirect contacts and susceptibility to viruses [72]. These host factors are those that influence any kind of virus infection and therefore are not specific for recombination. However, the distance between inoculation sites is of primary importance. Recombination occurs preferentially when high doses of both viruses are administered at the same site, as seen with PrV and BoHV-1 [17,73]. Cell factors, such as interferon and other chemokines, cell sensitivity and permissivity, and cell cycle are not further addressed here [74]. The rest of the review will therefore deal with viral factors, some of them being closely linked with cell factors (Figure 5).

(i) Dose of inoculated virus: *in vivo* recombination between two non-virulent PrV strains is dose-dependent [73]. High multiplicity of infection increases the rate of genetic recombination between PrV strains [75]. Similar doses of each virus avoid dominance of one parental strain.

(ii) Time interval between inoculation of the first and the second virus: an interval of 2 hours between infections allows *in vivo* recombination between PrV strains [73]. Only simultaneous or closely separated infections (maximum 4 hours) with two distinguishable BoHV-1 viruses lead to the production of a large amount of recombinant viruses *in vitro* [57] (Figure 6). Also with PrV, a very small time window with a maximum time interval of 4 hours is open for productive double infections [76].

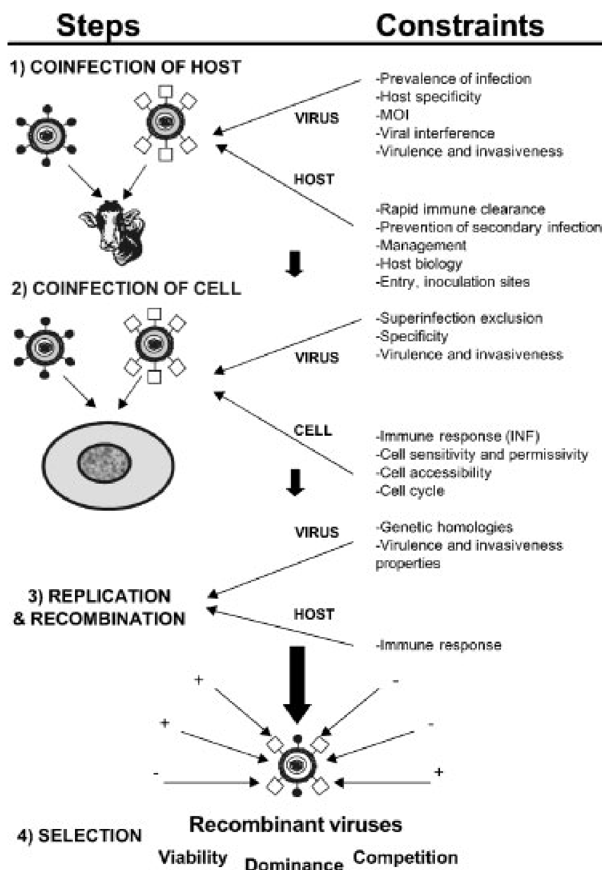

Figure 5. Production of viable recombinants and possible constraints. Virus and host constraints could block recombination by limiting or preventing the completion of any of the four steps

(iii) Distance between the marker mutations influences recombination [10]. However, in PrV, distance does not play a prominent role. Recombination between mutations of 266 bp apart in the thymidine kinase (TK) gene frequently occurred *in vivo* [73]. The same recombinant viruses were isolated *in vitro* as frequently as those issued from recombination between TK and glycoprotein gE genes which are separated by approximately 60 kbp [77].

(iv) Genetic homology: a high degree of genetic homology is one of the most important factors influencing homologous recombination. Indeed, as already stated, recombination was not detected between less closely related alphaherpesviruses, such as between HSV-1

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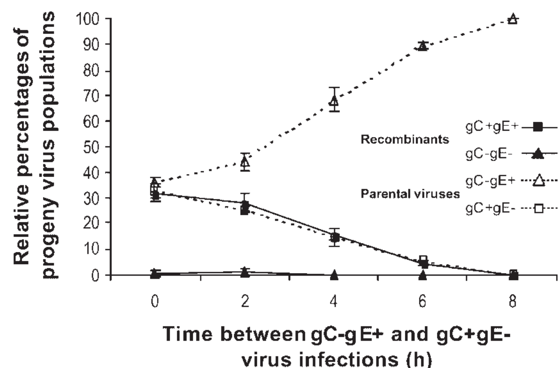

Figure 6. The effect of the time interval on recombination can be exemplified by the following experiment. MDBK cells were either coinfected with a bovine herpesvirus 1 (BovHV-1) gC- mutant (gC-/gE+) and a gE- mutant (gC+/gE-) or infected with BoHV-1 gC- mutant and superinfected with the gE- mutant, 2, 4, 6 and 8 h after infection with Lam gC- at a multiplicity of infection of 10. The rate of recombinants in the progeny virus population decreases with time until 8 h when they are no longer detected (from [57], with permission of American Society for Microbiology)

and PrV [67] or between BoHV-1 and CpHV-1 [16].

(v) Virulence: a difference in virulence and invasiveness between two coinfecting strains biases the temporal course of viral replication and spread in favour of the most virulent strain. In experiments of coinfection of neurons with PrV recombinants, independent of the infectious dose, the most virulent PrV strain tended to suppress the replication and spread of the less virulent one [78].

(vi) Latency: alphaherpesviruses can establish, in their specific hosts, a latent infection that could influence recombination *in vivo*. Indeed, recombination can occur during primary coinfection, but also after reactivation and re-excretion of one or both viruses, although recombination is unlikely during the latency phase. Latency increases the likelihood of cellular coinfection, which enables recombination between viruses. Nevertheless, as marked differences in invasiveness and virulence have been described between viruses, different patterns of distribution of viral DNA in tissue can occur and probably influence this likelihood of coinfection. Moreover, a single host can support the latent infection of two distinguishable strains of BoHV-1 [79] and a single neuron can be dually infected with two different alphaherpesviruses, such as HSV-1 and VZV [80]. Additional investigations are required to assess the impact of latency and its consequences on recombination.

VIRULENCE OF RECOMBINANT VIRUSES

Recombination may modify the virulence of alphaherpesviruses. In nature, the virulence of any given viral strain is most likely the result of the effects of the expression of a variety of viral genes. How these genes and their products interact with each other and with host proteins determines the outcome of the disease [81,82].

A natural BoHV-1 recombinant deleted in the gene encoding glycoprotein E was isolated *in vitro* [21]. It produced smaller plaques *in vitro* and was the basis for the development of a live attenuated marker vaccine against infectious bovine rhinotracheitis (IBR), the disease caused by BoHV-1. By multiple passages in cell culture, this strain arose from an illegitimate recombination between the S segment of two isomeric forms of the BoHV-1 genome. Such an event within the S segment was also obtained by spontaneous recombination between isomeric forms of PrV [83]. The rearrangement within the S segment of alphaherpesviruses most likely accounts for the differences found in length and gene content in this segment [84]. Coinfections could show contradictory effects. Indeed, although the most virulent parental or recombinant virus is usually favoured in the progeny population, the deleted viruses could be partly conserved due to complementation in cells coinfected by a glycoprotein deleted virus and a second, wildtype virus providing the missing glycoproteins [17].

In vivo studies reported generation of virulent recombinant HSV-1. Virulent recombinants can be generated following coinoculation of mice with two avirulent strains of HSV-1 [19]. Mixed infection with two avirulent strains of HSV-1 can result in synergistic increases in the severity of ocular or central nervous system virulence, primarily through the generation of recombinant viruses [59,81,85–87]. This is not always the case: interspecific recombinants between virulent HSV-1 and HSV-2 lost the parental phenotype [88].

Virulent PrV recombinants were obtained following *in vivo* coinoculations of mutant strains in mouse, sheep and swine, its natural host [73,89–91]. Recently, the rise and survival of recombinant wildtype BoHV-1 were detected in calves coinoculated with two mutant strains [17]. Recombinant

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BoHV-1 can establish a latent infection, is able to reactivate and therefore can be disseminated among animals. The consequences of the spread of recombinant viruses must be addressed cautiously. Other examples will be considered in the next section on vaccines.

The possible generation of chimeric genomes following recombination between alphaherpesviruses and other viruses cannot be excluded. This recombination event could result in the acquisition of new genes or regulatory elements that could adversely modify virulence. A recent example is the generation of chimeric molecules having sequences of Marek's disease virus (gallid herpesvirus 2) and avian leucosis virus subgroup J used for experimental coinfection of chickens. This provided evidence for avian retroviral inserts in the alphaherpesvirus genome [92].

ASSESSMENT OF RECOMBINATION FOR THE BIOSAFETY OF ANTIVIRAL THERAPY, HERPESVIRUS VECTORS AND VACCINES

Antiviral therapy

Alphaherpesviruses may acquire resistance against antiviral drugs by mutations in the genes encoding the target enzyme. These mutations can therefore be exchanged by recombination between viruses. Early works on acyclovir, a nucleoside analogue inhibitor, reported the role of recombination in HSV-1 to acquire either resistance or sensitivity to the antiviral. Recombination was also used to demonstrate the cooperative effects of two acyclovir resistance loci, situated in the TK and the DNA polymerase genes [93,94].

Viral vectors

Among alphaherpesviruses, HSV-1 is currently the most extensively engineered herpesvirus for purposes of gene or cancer therapy, especially for gene transfer into the brain.

Biosafety is regulated by the selection of a viral vector that is either attenuated for the host or unable to replicate in the host although still able to express the heterologous gene of interest. Several systems have been developed in which the vector is deleted for essential genes and produced in cells that complement the missing function. The use of a vector that has extensive deletions can reduce but not abolish the occurrence of reversion by recombination with wildtype viruses [95].

Humans and animals are often naturally infected with alphaherpesviruses which can reactivate sporadically. This opens up the possibility that a replication incompetent herpesvirus based vector, carrying a transgene for use in gene therapy procedures, may coinfect cells with the harboured wildtype virus, allowing a replication competent virus carrying the transgene to be generated. A further potential risk in using herpesvirus based vectors for gene delivery is that inoculation of individuals with a disabled vector could stimulate reactivation and recombination with latent wildtype virus already present in the individual. Therefore, assessment of the potential for recombination events between replication incompetent vectors or conditionally replicating vectors and wildtype viruses is of essential biosafety concern. Experiments have been performed to assess this risk in the nervous system. Upon intracerebral administration of replication defective HSV-1 to latently infected rats, reactivation of wildtype HSV-1 from latency has not been observed [96].

Vaccines

In human medicine, live attenuated herpesviral vaccines are not favoured, with the exception of the vaccine against varicella-zoster. There is no reported evidence of recombination between the Oka vaccine strain and a wildtype VZV strain. Some prototype VZV(Oka)-based recombinant live viral vaccines were generated by inserting Epstein-Barr virus glycoprotein gp350/220 in the VZV TK gene [97] or by inserting HSV-2 gD in the VZV S segment [98]. Live attenuated HSV vaccines have been developed as candidate vaccines. Experiments in mice showed that a potential *in vivo* recombination with a circulating wildtype HSV-1 strain could occur [87]. Moreover, two prototype attenuated vaccines (R7017 and 57020) derived from HSV-1 strain F have been developed by specific mutations. These vaccines showed attenuation in animal models but could establish latency and subsequently reactivate [99]. Therefore the risk of recombination *in vivo* exists but has not been seen yet.

Disabled infectious single cycle (DISC) herpesvirus vaccines contain genetically disabled virions that lack gene coding for an essential glycoprotein

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like gH, necessary for viral entry into the cell [100,101]. To compensate for this deficiency in the first infectious cycle, the disabled virus is grown in cells expressing the missing gene. DISC-HSV-1 and HSV-2 were evaluated as vaccines in animal models [102–104]. Although DISC-HSV-1 and 2 were shown to be safe, risks of recombination and reactivation with herpesviruses already infecting the host cannot be excluded theoretically.

The main concern currently is in veterinary medicine, where control programmes for several diseases are based on the massive use of live-attenuated vaccines. Especially two alphaherpesvirus infections, Aujeszky's disease caused by PrV, and IBR caused by BoHV-1, are controlled by vaccines harbouring a marker that permits serological differentiation of vaccinated animals [105].

The deletion in the gene encoding glycoprotein E (gE), a non-essential glycoprotein in BoHV-1, provides a serological marker which easily differentiates between vaccinated and infected animals. The risk of recombination between the BoHV-1 gE negative marker vaccine and the field strain must be assessed. During IBR epidemic events, intranasal gE deletion vaccination is frequently carried out and consequently co- and superinfections with wildtype and gE negative vaccine strains of BoHV-1 can occur with the possibility of generating virulent viruses from which gE has been deleted. These recombinant viruses could endanger control and eradication programmes. Recently, the isolation in the field of a BoHV-1 strain with gE deletion, strengthened the requirement of this risk assessment [106]. The low likelihood of cellular coinfections in natural conditions and the fact that superinfection does not allow recombination if a time interval longer than 4 hours has transpired between the two infections suggest that recombination and its potential consequences are rare events. However, a single recombinant that retains virulence and acquires the gE negative phenotype is enough to severely impair control programmes based on vaccination.

Coinfection of the F2 vaccine strain of FeHV-1 with a wildtype strain produced a high frequency of recombinants *in vitro*. Furthermore coinfection of F2 and a genetically engineered TK-deleted strain yielded recombinant viruses showing the same restriction endonuclease pattern as the virulent strain from which the TK deleted strain was derived [62]. Avian alphaherpesvirus vaccines share the same risk. Indeed a virulent field isolate of infectious laryngo-tracheitis virus (gallid herpesvirus 1, GaHV-1) was found with a TK gene sequence similar to the sequence typically found in a virulent strain, whereas its glycoprotein G sequence was identical to that of the vaccine strain [107]. This highly virulent strain was suspected to be produced by *in vivo* recombination between virulent and vaccine strains of GaHV-1. In conclusion, risk assessment of recombination needs to be addressed as a preliminary condition for the use of alphaherpesviruses as vaccines or vectors.

CONCLUSION

Recombination appears to be an essential part of the replication process of alphaherpesvirus DNA, but also depends on DNA replication and virus multiplication to create new genetic combinations. Therefore recombination largely contributes to the genetic diversity of alphaherpesviruses. The *in vitro* data support the conclusion that homologous recombination is a very frequent phenomenon. Its role is nevertheless underestimated in natural conditions. Indeed, in epidemiological situations favouring coinfections, recombination probably occurs at a high frequency, but few surveys have been carried out to isolate recombinant viruses in the field.

Quantitative assessment of recombination can be envisaged by the study of mixed concatemers isolated from coinfected cells. The analysis of these intermediates of replication, in the early phases of the virus replication cycle, allows the bias introduced by invisible or non viable recombinants, and by dominance of a virus over recombinants to be avoided. The development of such methods will help to perform accurate biosafety evaluation of recombination, especially for testing exchange of resistance against new antiherpesvirus compounds and for the use of alphaherpesviruses as vectors in gene therapy and vaccination, and as live-attenuated vaccines.

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