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Biology and evolution of the endogenous koala retrovirus

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Abstract. Although endogenous retroviruses are ubiquitous features of all mammalian genomes, the process of initial germ line invasion and subsequent inactivation from a pathogenic element has not yet been observed in a wild species. Koala retrovirus (KoRV) provides a unique opportunity to study this process of endogenisation in action as it still appears to be spreading through the koala population. Ongoing expression of the endogenous sequence and consequent high levels of viraemia have been linked to neoplasia and immunosuppression in koalas. This apparently recent invader of the koala genome shares a remarkably close sequence relationship with the pathogenic exogenous Gibbon ape leukaemia virus (GALV), and comparative analyses of KoRV and GALV are helping to shed light on how retroviruses in general adapt to a relatively benign or at least less pathogenic existence within a new host genome. (Part of a Multi-author Review)

Keywords. Retrovirus, koala, endogenous, gammaretrovirus, evolution.

Introduction

Endogenous retroviruses and retroviral elements are a ubiquitous feature of vertebrate genomes. They have been found in all genomes studied to date and make up a significant percentage of the total genomic sequence in species where they have been studied in detail. For example, up to 8–10 % of the human and mouse genomes are thought to be of retroviral origin [1]. In many cases these sequences have been found to be closely related to those of exogenous (horizontally transmitted) viruses but are mostly inactive. Despite their apparent universal presence in vertebrate genomes, the process of initial retroviral colonisation (endogenisation) of a wild species genome has not yet been observed [1].

Koala retrovirus (KoRV) was originally isolated as part of an investigation into the cause of leukaemia, lymphoma and immunosuppression in koalas [2] (Fig. 1). Exogenous retroviruses are known to cause these syndromes in other species, with the best-studied examples being retroviruses of cats and mice [3]. KoRV was found by sequence analysis to be closely related to a pathogenic exogenous virus of gibbons, gibbon ape leukaemia virus (GALV) – so closely related in fact, that these viruses appear to be conspecific (derived from a common ancestral virus) and may have arisen via a recent species jump between koalas and gibbons, most likely via a rodent intermediate [2]. However KoRV is an endogenous virus, albeit a very active one and linked to disease in its host. Recent studies have demonstrated that KoRV most likely entered its host within the last 200 years and is still undergoing the process of endogenisation [4]. It still displays many of the features of an exogenous virus such as the ability to produce infectious virus particles and variability in proviral copy number and sequence [4]. But when compared with GALV it appears to have undergone some modifications that reduce its replication efficiency in murine and human cells [5, 6]. Other work has

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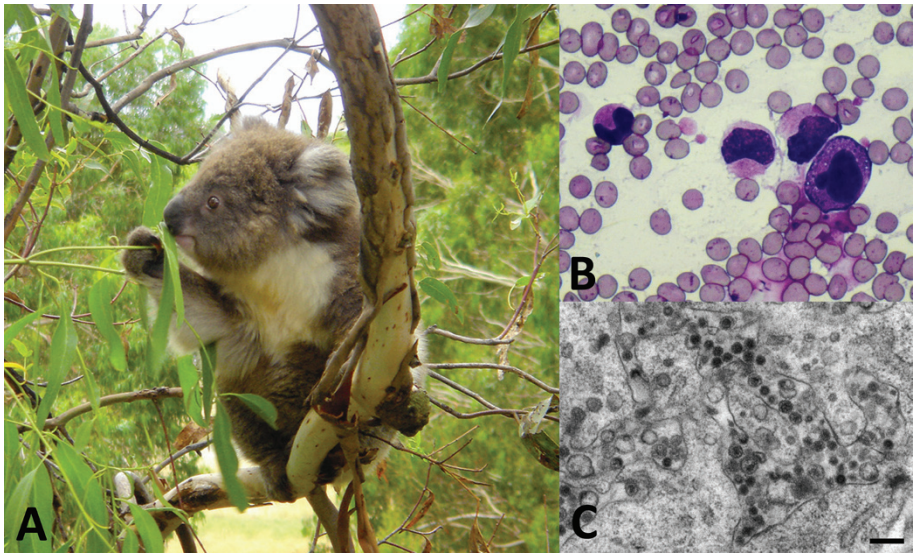

Figure 1. (*A*) Juvenile koala photographed on Raymond Island. (*B*) Peripheral blood smear from a leukemic koala showing typical neoplastic myeloid cells. (*C*) Electron micrograph of a thin section of bone marrow from a leukemic koala with enveloped viral particles showing typical central dense cores. Scale bar is 250 nm.

indicated that KoRV is capable of productive infection in rats and may have inherent immunosuppressive properties [7]. Overall, this group of viruses is beginning to provide early clues as to how the endogenisation process occurs, but much remains to be clarified.

Endogenous retroviruses

Retroviruses insert DNA copies of themselves into the host cell genome as part of their natural lifecycle. A consequence of this unusual lifecycle is that if viral genes become inserted into the host's germ line tissue, they can become inherited. This has led to the existence of two classes of retrovirus – exogenous, horizontally transmitted viruses and endogenous inherited viruses [8].

Endogenous retroviruses comprise genetic elements with a recognisable version of the classic retroviral structure [LTR-*gag/pro/pol/env*-LTR] that is integrated into the germ line cells of the host organism [8]. As noted above, they are present in all vertebrates studied to date, including birds, reptiles, marsupials, placental mammals and fish [9, 10]. In most cases they are very ancient insertions and have co-evolved with or perhaps even triggered speciation within their hosts [9]. The more extensively studied host species contain multiple groups of endogenous retroviruses from a number of retroviral genera.

Many ancient endogenous retroviruses are almost unrecognisable, missing entire genes or with highly divergent LTR regions. More recently endogenised retroviruses exhibit a higher degree of similarity to exogenous viruses, and indeed some remain functional [8]. In general, endogenous retroviral inserts are conserved across all members of a species. More variation is seen in the modern endogenous retroviruses; however, members of individual breeds or sub-strains usually have a highly conserved complement of endogenous retroviral elements [8, 11, 12].

Phylogenetic studies of endogenous and exogenous retroviruses have demonstrated that related retroviral elements are found in similar species [9, 13]. This indicates that retroviruses have in general co-evolved with their hosts. There are, however, exceptions to this, including that between KoRV and GALV, between RV-Echidna (an endogenous retrovirus of echidnas) and SNV (exogenous spleen necrosis retrovirus of ducks), and between RD114 (an endogenous virus of cats) and BaEV (baboon endogenous retrovirus) [9, 14]. Where similar viruses exist in such diverse species, there is presumed to have been a cross-species transmission event. Separate analysis of endogenous retroviral *env* and *pol* genes that results in different phylogenetic clustering of viruses also indicates that some have probably arisen by recombination [13].

While endogenous retroviruses are usually inactive within the genome, some cell lines can be stimulated to produce infectious virus, and some strains of mice and chickens will spontaneously produce virus [8]. Suppression of replication of these viruses is affected by host cell methylation, though whether this is the cause or effect of inactivation is debatable [8, 15,16].

A retrovirus of koalas

KoRV was initially discovered during the course of an investigation of haematopoietic neoplasia in koalas [2]. This work followed earlier suggestions of a viral aetiology for koala lymphosarcoma [17, 18]. The

suggestion gained more credence with the finding by electron microscopy of gammaretrovirus particles in bone marrow from a leukaemic koala [19]. This was followed by a similar report from the San Diego zoo of gammaretroviral particles in peripheral blood mononuclear cell (PBMC) cultures of koalas from a colony with a high incidence of neoplasia and opportunistic infection [20]. The tumours in this report were multicentric lymphoma, osteosarcoma of the long bones and osteochondroma of the bones of the skull. These workers also reported PCR amplification of a portion of a reverse transcriptase gene, though not its sequence.

Hanger et al. [2] reported gammaretrovirus particles by electron microscopy in mitogen-stimulated PBMC cultures from 163 of 166 koalas tested and lymphoma tissue from three koalas. They also reported the full nucleotide sequence of this novel gammaretrovirus, which they designated KoRV. KoRV was found to be a complete endogenous virus with intact *gag, pol* and *env* reading frames [2]. The virus also had intact 3′ and 5′ LTR regions, CAAT and TATA boxes and a poly A site, indeed all the components necessary for a replication-competent retrovirus. Provirus was detected by PCR in all koalas tested (17 animals) whether healthy or diseased and in multiple tissues tested by Southern hybridisation (2 animals), suggesting that KoRV was an endogenous retrovirus. RNA transcripts were present in the circulating white blood cell fraction and serum of all animals tested (10 animals). Reverse-transcriptase activity from PBMC cultures was also detected (9/32 animals), indicating that the virus was being actively transcribed [2].

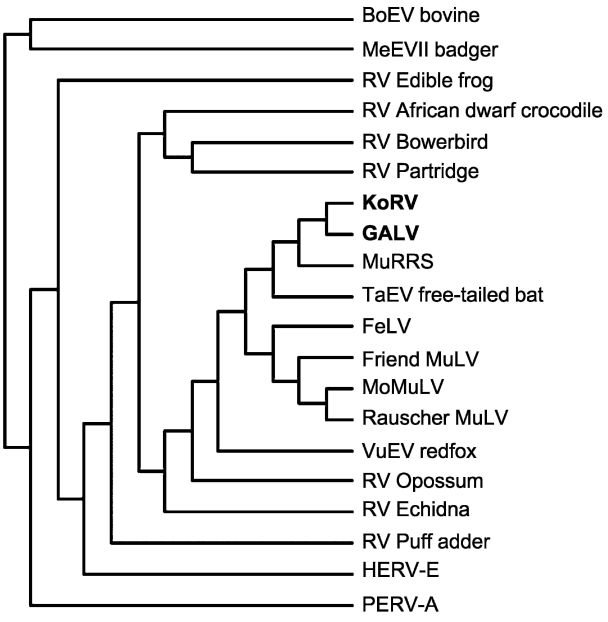

Figure 2. Neighbor-joining tree showing the phylogenetic relatedness of selected gammaretroviruses with the close relationship between KoRV and GALV highlighted in bold. Both exogenous and endogenous retroviral sequences were retrieved from sequence databases and aligned in Vector NTi. A stretch of 570 nucleotides from the reverse transcriptase coding region of each was then used to generate the tree. BoEV, X99924; MeEVII, X99927; RV edible frog, AJ236118; RV African dwarf crocodile, AJ236124; RV bower bird, AJ236114; RV partridge, AJ236125; KoRV, AJ236122; GALV, NC001885; MuRRS, X02487; TaEV free-tailed bat, X99933; FeLV, NC001940; Friend MuLV, NC001362; MoMuLV, NC001501; Rauscher MuLV, NC001819; VuEV redfox, X99935; RV opossum, AJ236123; RV echidna, Aj236114; RV puff adder, AJ236110; HERV-E, M10976; PERV-A, NC003059.

A close relationship with gibbon ape leukemia virus

KoRV displays a 78% similarity at the nucleotide sequence level to GALV, a pathogenic exogenous gammaretrovirus of gibbons [2]. Phylogenetic analysis also grouped KoRV and GALV together, separate from other gammaretroviruses (Fig. 2). Martin et al. [9] had previously sequenced part of the KoRV *pol* gene, calling it RV Koala, and had also noted the unusual similarity to GALV [9]. Two viruses with this degree of similarity are unlikely to have arisen independently, and this raises the possibility of a species jump having occurred, most likely via acquisition from a third species. The sequence similarity also suggests that they have diverged only recently – perhaps as recently as decades ago [21], a situation unprecedented in retroviral study where the most recent previously reported endogenisation event was a porcine endogenous retrovirus which is thought to have entered its host approximately 3.5 million years ago [22]. Given the evolutionary and geographical isolation of koalas and gibbons, a host jump is likely to have required an intermediate step, either through human iatrogenic spread or an intermediate host species.

GALV was first identified as an exogenous gammaretrovirus responsible for several epidemics of leukaemia and lymphoma in captive gibbon (*Hylobates lar)* colonies. The initial isolation of the virus and demonstration that it was the causative agent of haematopoetic neoplasia was in 1972 from the SEATO laboratory gibbon colony in Bangkok, Thailand [23–25]. Other disease outbreaks and virus isolations have occurred, including at the San Francisco Medical Center [26] and in an introduced free ranging population on Halls Island, Bermuda [27, 28]. There has also been an isolation of a virus complex including a replication-defective oncogene-containing virus along with a helper virus in a pet woolly monkey (*Lagothrix sp*) in California [29, 30]. Strains of the virus are named after the place, tissue or species they were first isolated, hence GALV-SEATO (which refers to iso-

lates obtained from a Southeast Asian Treaty Organization colony), GALV-SF (San Francisco), GALV-H (Halls Island), GALV-Br (brain tissue) and WMSV (woolly monkey sarcoma virus) [28]. There have also been several reports of the isolation of GALV strains from human cell cultures [31–33], although these are thought to have been laboratory contaminations rather than virus infection of the original human tissues.

GALV has been shown to be transmissible to juvenile gibbons, resulting in granulocytic leukaemia, anaemia and proliferative bone lesions [25]. Different strains of the virus cause myelogenous leukaemia or lymphocytic leukaemia, and these strains appear to be relatively stable on passage [34, 35]. However, this was determined using liquid hybridisation techniques, and the exact nature of these differences has not been fully described. In experimental studies, gibbons that mount a neutralising antibody response do not develop disease, whereas those that do not produce antibody go on to become persistently viraemic and develop disease [26]. In serological surveys of captive colonies it appears that a low percentage of apparently healthy gibbons are seropositive for GALV [24, 36]. Animals affected by disease range in age from 3.5 to 8 years, the disease course lasting from 3 months to 3 years. GALV is shed in urine and faeces, but its exact mode of transmission is uncertain [36].

Natural transmission of the virus has been known to occur both pre- and post-natally, with animals infected *in utero* developing higher proviral loads. It is possible that the animals infected *in utero* had endogenised the virus, as it was detected in muscle tissue by liquid hybridisation techniques, though this signal may have been due to blood contamination [37]. Animals infected post-natally only have detectable virus in a limited range of tissues, and uninfected or antibody-positive animals do not demonstrate viral inserts in tissues by Southern blotting or molecular hybridisation [37, 38]. Interestingly, there is some evidence for truncated GALV proviral inserts in tumour tissues, though there has been no study on the significance of these findings [38].

The SSV/SSAV (simian sarcoma/simian sarcoma-associated virus now known as woolly monkey sarcoma virus or WMSV) complex of viruses has been isolated only once from a male woolly monkey (*Lagothrix* sp.) suffering from multiple fibrosarcomas, myelofibrosis and myeloid metaplasia presented to a veterinary clinic in the United States [29]. It is very likely that the monkey contracted the virus from a gibbon it was housed with that had died from leukaemia [30]. Early studies of this virus complex relied on methods such as antigen-based radioimmunoassay and restriction enzyme digest mapping to demonstrate the similarity of GALV and WMSV [39–41]. However, with the advent of molecular techniques and routine sequencing techniques, it was revealed that SSV was a replication-defective virus containing an oncogene (v-sis) replacing part of the envelope gene of SSAV (the replication-competent component of the complex). This oncogene is responsible for the rapid cell transformation characteristic of this virus [42]. Sequencing of the GALV SEATO strain revealed it to be identical to partial sequences of the non-oncogene components of SSAV [30]. The GALV SEATO strain has a classic minimal gammaretrovirus sequence without any oncogenes or any accessory proteins.

Further studies on GALV have focused on its receptor usage. GALV is known to infect a wide variety of cell lines, including human cell lines, though its potential for zoonotic transmission has not been fully explored. Its cellular receptor is known to be the ubiquitous sodium-dependent phosphate transporter (PiT 1) [43–45]. The wide species and cellular tropism of GALV has led to its popularity in trials of potential vectors for gene therapy [31, 43, 46–48].

Retroviruses of Asian rodents

Endogenous retroviruses antigenically related to GALV have been isolated by co-cultivation of cell lines from Asian rodents (*Mus caroli, Mus cervicolor* and *Vandeleuria oleracea*) with more standard cell lines. There has been a suggestion that GALV may have arisen from cross-species transmission of these viruses [49–51], as hybridisation studies indicate that they are probably endogenous elements that are closely related to GALV, and to each other [52, 53]. Two separate viruses have been isolated from *Mus cervicolor* [52], and the receptor usage of one of these, M813, has been studied in more detail. This isolate has been shown to have a novel receptor usage when compared with other MuLVs [54] and induces a T-cell lymphoma when inoculated into laboratory mouse (*Mus musculus*) strains DBA/2J or DDD [55]. A more recently identified endogenous virus has been isolated from cultured tail fibroblasts of *Mus dunni* [56] and has been characterised as having a very wide potential host cell range reflecting a different receptor usage to other gammaretroviruses [57]. The full sequence of this virus has been determined, and it has been shown to have a simple gammaretroviral structure. The LTR regions are closely related to murine VL30 elements, while the coding sequences are most closely related to GALV, though the phylogenetic analysis presented in this paper is not extensive [58]. The sequence of the other Asian rodent viruses has not been determined, and

no studies have been performed as to the prevalence of these viruses in wild populations. However, they remain the most likely candidates for the origin of GALV and KoRV.

A newly endogenised virus

As described above, KoRV was originally thought to be an endogenous retrovirus, albeit a very active one. Our work confirmed that KoRV was linked to hematopoietic neoplasia in koalas [59]. The production of gammaretroviral particles from lymphoma tissue of koalas was observed, and using real-time PCR measurements of viral RNA levels in blood as a marker of viraemia, a statistically significant relationship between high viral load and neoplasia was found. A trend towards high viral load being associated with clinical chlamydial disease was also seen. Chlamydiosis is accepted as the major disease problem in koala populations, causing ocular and urogenital infections; however, many animals carry the organism without pathogenic consequence. Expression of disease is thought to be due in part to an underlying immunosuppressive state, a known consequence of retroviral infection. It has been postulated that the high levels of KoRV viraemia may be directly responsible for the induction of this immunosuppression in koalas [4]. Indeed, the viral loads in some koalas were extreme and resemble those found in cats with end-stage disease caused by FeLV. The disease associations of the virus and the high level production of viral particles are typical features of exogenous viruses [59], suggesting that KoRV in some respects behaves more like an exogenous virus than an endogenous one. Despite these exogenous characteristics, KoRV was definitively demonstrated to be an endogenous virus in south-east Queensland koalas [4]. The virus was shown to be present in koala sperm by single-cell fluorescent *in situ* hybridisation (FISH) and single-cell PCR and to be inherited as a normal Mendelian allele by Southern blotting of blood cell DNA from a group of related animals. Banding patterns in offspring were shown to be a combination of parental patterns, with some bands inherited across three generations. However, it was also demonstrated that KoRV is not yet a fixed endogenous retroviral element, as proviral copy number and sequence varied markedly between individual animals as did the number and pattern of proviral insertions [4]. FISH performed on chromosome spreads derived from koala PBMCs revealed proviral inserts scattered throughout the koala genome [4]. The proviral insert pattern on chromosome spreads from different koalas appeared to vary markedly, consistent with the Southern blotting data [60].

Koalas in southern Australia have a unique history, having been nearly exterminated by hunting pressure in the late 19th century. In South Australia and parts of Victoria the species was made locally extinct and was only preserved by the establishment of island populations which were then used to restock the mainland. These populations are mostly descended from the colony established on French Island in the Western Port district of Victoria in the 1890s [61]. The study by Tarlinton et al. [4] found that animals from one of these island populations (Kangaroo Island) that had been isolated since the 1920s appeared to be free of KoRV, while koalas in the southern state of Victoria were found to have a mixed prevalence of KoRV proviral insertions. This epidemiological pattern strongly suggests a spreading disease front. When combined with the earlier phylogenetic analysis, it strongly suggests that KoRV has entered the koala population only within the last 200 years and is still undergoing endogenisation.

Infectious KoRV has also been isolated from koala peripheral blood mononucleocytes co-cultured with a variety of standard cell lines [7]. It was found to replicate in human 293 kidney cells, human T-lymphocyte cell lines C1866 and CEM, and rat fibroblast cells (rat 1) [7]. The virus isolate was used to infect Wistar rats with productive infection demonstrated by proviral integration, viral protein (*env*) detection, increasing viral load measured by real-time PCR and recovery of infectious virus from rat PBMCs. The rats ultimately controlled the infection, with no virus detectable after 63 days. One rat in this study developed fibrosarcoma. However, it is unclear whether this was linked to KoRV infection. This study clearly demonstrated that KoRV is able to replicate as an exogenous virus and productively infect rodent hosts, lending credence to the rodent intermediate host theory. They also reported the induction of neutralising antibodies in rats immunised with the recombinant ectodomain of the KoRV transmembrane (TM) protein p15, a component of *env* (Fig. 3), suggesting that the development of vaccination strategies aimed at preventing the spread of the virus in isolated KoRV-free koala populations may be possible. It has been suggested that retroviral-induced immunosuppression in koalas plays a key role in the high incidence of opportunistic infections such as chlamydiosis [2]. Feibig et al. further showed that when added to human PBMCs in culture, purified KoRV was able to stimulate the increased production of IL-10, IL-6 and MCP-1, known markers of lymphocyte proliferation inhibition [7]. They proposed that this was a consequence of the activity of a potential immunosuppressive domain (ISD) within the TM protein (Fig. 3). This region of the retroviral

envelope gene product is highly conserved, as it is embedded within the region of TM that plays a critical role in the formation of the post-fusion coiled-coil structure that drives viral and host cell membrane fusion. The ISD is found within the TM domain of many retroviruses and consists of a small 17-amino acid sequence which is thought to be directly immunosuppressive by as yet unknown molecular mechanisms [62]. Further studies will be necessary to confirm whether this ISD, in its native configuration contributes to the underlying immunosuppression of koalas endogenised by KoRV.

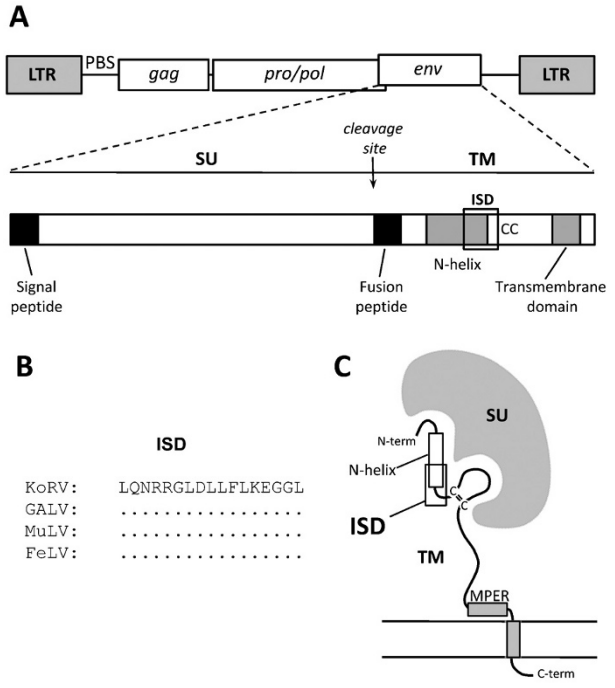

Figure 3. Schematic of a simple gammaretrovirus. (*A*) genome organization and selected features of the *env* gene product; PBS, primer binding site; SU, surface unit or gp70; TM, transmembrane or p15; CC, disulphide-bonded loop; ISD, putative immunosuppressive domain [62]. (*B*) KoRV sequence corresponding to the 17 amino acids defined as representing the ISD [62]. Peptides derived from the equivalent region of MuLV and FeLV (dots represent identical residues) have been shown to be immunosuppressive [62,7]. (*C*) Schematic of the SU/TM structure anchored in the viral membrane. MPER, membrane proximal ectodomain region. The location of the putative ISD sequence is shown. This domain begins within the N-helix, which is responsible for *env* trimer formation, and ends at the beginning of the disulphide-bonded loop region, which provides the core non-covalent interactions between SU and TM on the mature virion.

Oliveira et al. [5] focused on KoRV envelope gene receptor usage in comparison with GALV. They created a KoRV/GALV chimeric *env* gene pseudotype and found that the KoRV *env* gene variable region sequences (variable regions A and B) affords viral entry into a variety of cell lines, including mouse (MDTF, SC1, NIH3T3, MMK), rat (NRK), human (293T and HOS), bovine (MDBK) and hamster (BHK, E36). This contrasts with the observation by Fiebig et al. [7] that KoRV did not replicate in NIH3T3 cells. The two groups, however, used different systems (wild-type KoRV versus KoRV pseudotyped vectors) to study the cell susceptibility of different KoRV isolates with slightly different sequences. Sequencing of wild-type KoRV envelope genes indicates considerable variation with a variety of viral *env* quasispecies present in individual animals [60]. It seems likely that different quasispecies may have different cell tropism and replication efficiencies as has been previously reported for HIV [63]. Oliveira et al. [5] also presented interference assay data suggesting that the KoRV/GALV chimera also uses the human and murine PiT1 as its receptor.

This same group compared the infectivity of the KoRV and GALV envelope proteins in pseudotyped MuLV constructs with KoRV or GALV *gag/pol* or *env* proteins substituted for the equivalent murine ones [6]. They reported a greatly reduced viral titre in transfected murine cells for constructs containing KoRV sequences when compared with those from GALV. Mutation of the GALV *gag/pol* late domain consensus sequence to that of the KoRV sequence reduced GALV *gag/pol* infectivity. Similarly, the KoRV *env* sequence showed decreased infectivity when substituted for the GALV sequence. Comparison of all 17 available KoRV [60] and 4 available GALV sequences showed mutations in a conserved CETTG motif (CETAG or CGTAG in KoRV) between the two viruses. This sequence is known to be present in exogenous but not endogenous murine gammaretroviruses. Mutation of this sequence in the GALV envelope to the KoRV sequence resulted in decreased cell fusion after exposure to virus when compared with the original GALV construct, confirming the key role of these residues. Substitution of the KoRV residues T86, L87, Q141, P142 and R143, which differ from the GALV receptor binding domain, also demonstrated a marked reduction in viral titre. These differences between viral sequences were proposed to be adaptive changes in KoRV, reducing pathogenicity and facilitating endogenisation [6]. Extending these pseudotype studies to those employing natural isolates of KoRV should reveal additional subtleties in KoRV-host cell interactions [60].

The KoRV/GALV group of viruses presents a unique opportunity to study the process of endogenisation of retroviruses in action. Studies with these viruses have already provided the first insights in to how simple mutations can drastically alter virus infectivity, perhaps reflecting the beginnings of reduced pathogenic-

ity of endogenous retroviruses. They have also provided tantalising hints as to how cross-species transmission may occur via third species intermediates (in this case rodents) that need to be pursued further. Study of the pathogenicity of the disease in wild koala populations as it spreads over time will provide invaluable information on whether both endogenous and exogenous forms of the virus co-exist and how both host and virus adapt and evolve to progress from the current situation of high levels of virus induced disease to a possible commensal arrangement.

There is also little known about GALV in wild gibbons or about the retroviruses of potential rodent intermediates. Characterisation of KoRV and GALV wild-type isolates and the rodent intermediates may also help to clarify the process of viral adaptation to endogenisation. The rodent model of KoRV replication could be explored for the endogenisation potential of KoRV as well as the pathogenicity of the virus and the potential for control of viral replication. The inactivation and control mechanisms that various hosts use, such as RNA silencing and the activity of TRIM family proteins, also warrant exploration.

Overall this group of viruses provides a unique opportunity to unravel some of the fundamental processes by which endogenous viruses enter their host species and how the hosts adapt to this viral assault. The large number of retroviral sequences littering vertebrate genomes indicates that they are likely to play a significant role in mammalian evolution. Many authors have speculated on whether these endogenous viruses have beneficial functions for their hosts or are just the remnants of the constant evolution between pathogen and host. The study of this group of viruses will hopefully shed light on this fundamental process in evolution.

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