Virology 475 (2015) 28–36

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Genomes and Evolution

Sequence variation of koala retrovirus transmembrane protein p15E among koalas from different geographic regions

Yasuko Ishida [a,*,1], Chelsea McCallister [b,1], Nikolas Nikolaidis [b,1], Kyriakos Tsangaras [c], Kristofer M. Helgen [d], Alex D. Greenwood [c], Alfred L. Roca [a,e,**]

[a] Department of Animal Sciences, University of Illinois at Urbana-Champaign, 1207 W. Gregory Drive, Urbana, IL 61801, USA
[b] Department of Biological Science and Center for Applied Biotechnology Studies, California State University, Fullerton, 800 North State College Blvd, Fullerton, CA 92834, USA
[c] Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, 10315, Berlin, Germany
[d] National Museum of Natural History, Smithsonian Institution, PO Box 37012, MRC 108, Washington, DC 20013, USA
[e] The Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

ARTICLE INFO

Article history:
Received 18 April 2014
Returned to author for revisions
4 September 2014
Accepted 28 October 2014
Available online 22 November 2014

Keywords:
Endogenous retroviruses
Epitopes
KoRV
Phascolarctos cinereus
Vaccines

ABSTRACT

The koala retrovirus (KoRV), which is transitioning from an exogenous to an endogenous form, has been associated with high mortality in koalas. For other retroviruses, the envelope protein p15E has been considered a candidate for vaccine development. We therefore examined proviral sequence variation of KoRV p15E in a captive Queensland and three wild southern Australian koalas. We generated 163 sequences with intact open reading frames, which grouped into 39 distinct haplotypes. Sixteen distinct haplotypes comprising 139 of the sequences (85%) coded for the same polypeptide. Among the remaining 23 haplotypes, 22 were detected only once among the sequences, and each had 1 or 2 non-synonymous differences from the majority sequence. Several analyses suggested that p15E was under purifying selection. Important epitopes and domains were highly conserved across the p15E sequences and in previously reported exogenous KoRVs. Overall, these results support the potential use of p15E for KoRV vaccine development.

Introduction

Endogenous retroviruses (ERVs) originate when exogenous retroviruses integrate into the genomes of host germ line cells (Boeke and Stoye, 1997; Bromham, 2002; Coffin, 2004; Stoye, 2012). ERVs are subsequently transmitted vertically in the host lineage, from parent to offspring through Mendelian inheritance (Boeke and Stoye, 1997; Bromham, 2002; Coffin, 2004; Stoye, 2012). The koala retrovirus (KoRV) is the only retrovirus known to be in the midst of transitioning from exogenous to endogenous form in its host species, the koala (*Phascolarctos cinereus*) (Tarlinton et al., 2006). Koala populations in northern Australia exhibit 100% prevalence of KoRV while in southern Australian populations many koalas are completely free of the virus. This suggests that KoRV initially affected koalas in northern Australia and is currently spreading to southern populations (Simmons et al., 2012; Tarlinton et al., 2006). Simmons et al. (2012) estimated that northern Australian koalas carry an average of 165 copies of KoRV per cell while southern Australian koalas carry 1.29×10^{-4} to 1.50 copies per cell (although some southern Australian koalas are completely free of KoRV). KoRV has been implicated in immunosuppression and is thought to be associated with leukemia, lymphoid neoplasia (Tarlinton et al., 2005), and chlamydiosis (Tarlinton et al., 2005). KoRV variants with more limited geographic distributions, perhaps reflecting more recent origins, have been reported recently (Shimode et al., 2014; Shojima et al., 2013a, 2013b; Xu et al., 2013); some sequence variants may be especially malignant (Shojima et al., 2013b; Xu et al., 2013). Many koala populations appear to have low genetic diversity especially in southern Australia (Cristescu et al., 2009; Houlden et al., 1996; Taylor et al., 1997; Tsangaras et al., 2012). Reduced genetic diversity can lower the ability of a population to resist pathogens (Spielman et al., 2004). The development of vaccines against KoRV may be an option for maintaining KoRV-free koala populations and to protect koalas in zoos.

KoRV is a gammaretrovirus related to the gibbon ape leukemia virus (GALV) (Hanger et al., 2000). Immunization and vaccine experiments have been conducted on various gammaretroviruses.

* Corresponding author. Tel.: +1 217 333 4679 (office).
** Corresponding author at: Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA. Tel.: +1 217 244 8853 (office).
E-mail addresses: yishida@illinois.edu (Y. Ishida), chelseam@csu.fullerton.edu (C. McCallister), nnikolaidis@exchange.fullerton.edu (N. Nikolaidis), ktsangaras@gmail.com (K. Tsangaras), helgenk@si.edu (K.M. Helgen), greenwood@izw-berlin.de (A.D. Greenwood), roca@illinois.edu (A.L. Roca).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.virol.2014.10.036
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For porcine endogenous retrovirus (PERV) and feline leukemia virus (FeLV), the transmembrane (TM) protein p15E and the surface (SU) protein gp70, which are parts of the viral envelope, have been tested for their potential for immunizing the host species (Denner et al., 2012; Kaulitz et al., 2011; Langhammer et al., 2011; Waechter et al., 2013). Gammaretrovirus envelope proteins are less variable than those of HIV-1 (Denner, 2013; Mansky and Temin, 1995). Immunization studies with the envelope proteins of gammaretroviruses PERV, FELV and KoRV across mammalian species have consistently obtained neutralizing antibodies. When both p15E and gp70 envelope proteins have been used for immunization against PERV in various animal species such as goats and rats, the resulting immune response and protection proved more efficient than immunizing with a single protein (Denner et al., 2012; Kaulitz et al., 2011). Epitope mapping of the p15E envelope protein of PERV and FeLV has identified two epitopes that are highly conserved among gammaretroviruses and are targets for host antibodies (Fiebig et al., 2003; Langhammer et al., 2006). Epitope E1 is in the fusion peptide-proximal region (FPPR), while epitope E2 is in the membrane-proximal external region (MPER). The epitopes in p15E of PERV and epitopes in gp41 (which corresponds to p15E) of HIV-1 are structurally and functionally similar (Fiebig et al., 2003). It has been experimentally shown that the MPER of HIV-1 contains at least 3 epitopes recognized by antibodies 2F5, 4E10, and 10E8 (Huang et al., 2012; Stiegler et al., 2001; Zwick et al., 2001, 2005). Similarly, in a recent study of PERV in which affinities of antibodies were tested for both MPER and FPPR, antibodies directed against MPER (E2) were found to be involved in neutralization (Waechter and Denner, 2014).

In addition to E1 and E2, p15E contains an immunosuppressive domain that is highly conserved among different retroviruses including the human immunodeficiency virus (HIV) (Denner et al., 1994). The protein p15E also contains the heptad repeat 1 (HR1) and heptad repeat 2 (HR2) motifs, which mediate viral protein-protein interactions and are important for viral fusion (Eckert and Kim, 2001; Skehel and Wiley, 2000). Additionally, the p15E protein contains the homotrimer interface, a domain that mediates the formation of a trimer structure responsible for viral fusion with the target cell and infection (Eckert and Kim, 2001; Shu et al., 1999); a chloride ion (Cl) binding site that stabilizes the trimeric structure (Lamb et al., 2011); and a CX(6)C motif that forms an intra-subunit disulfide bond in some viruses (Kobe et al., 1999; Wallin et al., 2004). The high degree of conservation of domains and of epitopes make the p15E polypeptide a good candidate to consider for targeting by neutralizing antibodies (Denner and Young, 2013).

In the case of KoRV, p15E-specific neutralizing antibodies have been shown to efficiently lower provirus integration (Fiebig et al., 2006). Thus the KoRV p15E polypeptide may be a potential target for vaccine development. Despite the important functional domains and motifs of the KoRV p15E region, its sequence variation or conservation have not been well characterized to date, especially for KoRV in southern Australian koalas. In this study, we examined the degree of sequence variation in KoRV p15E in four koalas of different geographic origins as well as among sequences published by previous studies (Avila-Arcos et al., 2013; Fiebig et al., 2006; Hanger et al., 2000; Shojima et al., 2013a; Xu et al., 2013). We also investigated the effects of this variation on the structure and function of this biologically and clinically important KoRV region by conducting protein structure modeling.

Results

Identification of protein epitopes and functional residues

The position of the coding region for p15E in KoRV (GenBank: AF151794) (Hanger et al., 2000) was determined based on homology to gibbon ape leukemia virus (GALV; GenBank accession number NC_001885) (Delassus et al., 1989) and feline leukemia virus (FeLV; GenBank NC_001940) (Chen et al., 1998). KoRV p15E corresponded to positions 7295 to 7885 (591 bp) of AF151794 (Hanger et al., 2000). The p15E polypeptide thus consists of 197 amino acids at positions 464 to 660 of the envelope protein. Homology to these other retroviruses allowed us to estimate that epitope 1 in KoRV would consist of amino acids 492 to 498 of Env (29 to 35 in p15E), epitope 2 would consist of amino acids 592 to 597 (129 to 134 in p15E), while the immunosuppressive domain would consist of amino acids 533 to 549 (70 to 86 in p15E). Amino acids for both E1 and E2 were highly conserved between the reference KoRV sequence and GALV (NC_001885) (Delassus et al., 1989). When KoRV was aligned to PERV (AM229312) (Preuss et al., 2006), E2 showed a higher degree of conservation than E1 among KoRV, GALV, and PERV (Fig. 1). A high degree of conservation for the immunosuppressive domain was also evident in an alignment across retroviruses, as in this region there were no amino acid differences among KoRV, GALV and PERV (Fig. 1). The Conserved Domain Database (CDD) of NCBI was used to identify conserved regions and to infer the functional amino acids by homology to other retroviruses. Both E2 and the immunosuppressive domain were verified as being conserved. The CDD was also used to determine the position of the remaining motifs present on the p15E region of KoRV. This analysis revealed that the HR1 and HR2 motifs were located at positions 33–78 and 103–112, respectively. The Cl binding site was the asparagine at position 72

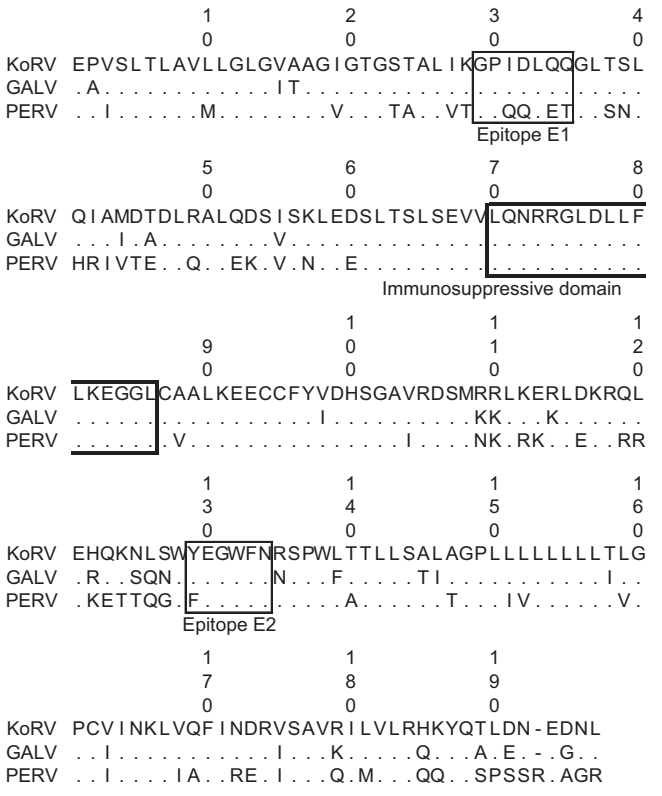

Fig. 1. Amino acid alignment of p15E in KoRV, GALV and PERV. The amino acid residues are shown for p15E-1, which was used as the reference KoRV. Amino acid residues in the other viruses that match the reference are indicated by dots; residues that differ from the reference sequence are shown; dashes indicate a shorter length for a polypeptide at the C-terminus. The immunosuppressive domain and epitopes are indicated by boxes; the immunosuppressive domain is fully conserved and E2 is highly conserved across the retroviruses. The two epitope positions were estimated in KoRV and GALV by homology with the epitopes in PERV (Fiebig et al., 2003). The position of the immunosuppressive domain was also determined by homology to other retroviruses (Denner et al., 1994). The amino acid sequence shown for GALV is based on translation of GenBank accession NC_001885 (Delassus et al., 1989); for PERV the translation is for PERV-C, GenBank accession AM229312 (Preuss et al., 2006).

(conserved among many retroviruses), the CX(6)C motif was at position 87–94, and the homotrimer interface was composed of several amino acids found at positions 43–112 (Fig. 2).

DNA and amino acid sequence variation of p15E

We examined the diversity of the KoRV coding region for p15E in one northern Australian koala (Pci-SN404) kept at a North American zoo and three southern Australian koalas (Pci-157 from Stony Rises, Pci-128 from Brisbane Ranges, and Pci-182 from Kangaroo Island). In total, 172 clones were sequenced across four koalas. Nine sequences among the 172 were not in frame. One clone in koala Pci-SN404 had a mutation that coded for a stop codon. Two clones of Pci-128 had a deletion at nucleotide position 84 of p15E that would cause a frameshift and a premature stop codon. Another frameshift mutation that did not cause a stop codon was detected in six clonal sequences of Pci-157. The frameshift was caused by a 19 bp deletion beginning at position 553 of

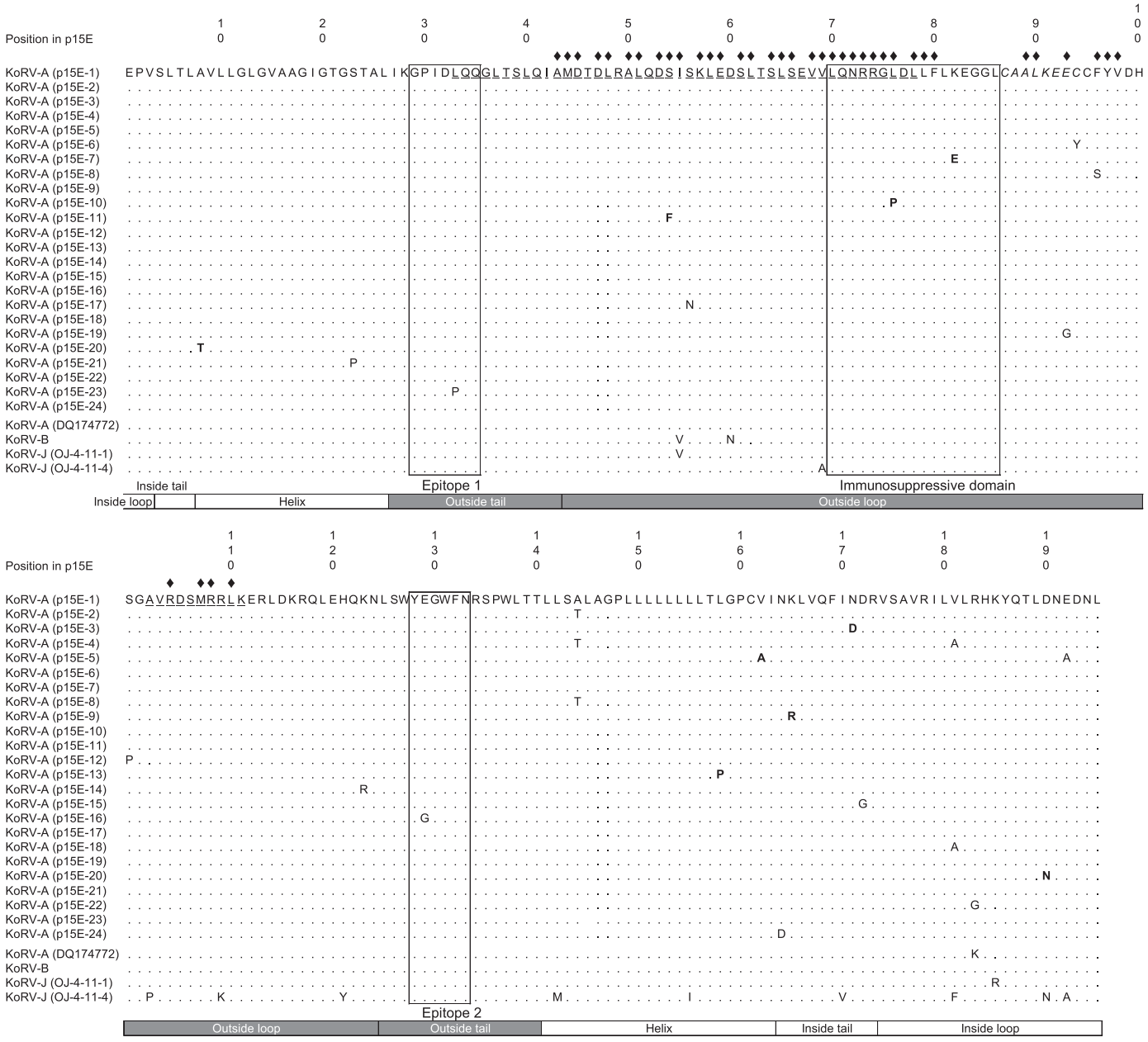

Fig. 2. Amino acid variation inferred from 163 KoRV p15E DNA sequences. DNA from 4 koalas was amplified and cloned. Each clone included the complete codon sequences of p15E. The alignment shows the inferred amino acid sequences, with the translation of the most common haplotype (p15E-1) used as a reference for the alignment. Positions are numbered relative to the start of p15E; dots indicate matches to the reference. Among the 163 clones sequenced that had intact open reading frames, there were 39 distinct DNA sequences, of which 16 coded for the amino acid sequence of p15E-1 (i.e., they differed only by synonymous mutations). Most of the KoRVs in Genbank (JQ244835 to JQ244839, AF151794 and AB721500) (Avila-Arcos et al., 2013; Hanger et al., 2000; Shojima et al., 2013a) coded for the same amino acids as p15E-1. The amino acid differences are shown for those that did not code for the same residues: KoRV-A GenBank accession DQ174772, KoRV-B NC_021704, KoRV-J OJ-4 clone 11-4 AB822553, and KoRV-J OJ-4 11-1 (clones 11-1, 2, and 5 had identical sequences, and correspond to Genbank accessions AB823238, AB828004, and AB828005, respectively). Amino acid residues for p15E-2 through p15E-24 represent translations of newly generated KoRV sequences that had non-synonymous mutations. The positions of epitope E1, epitope E2, and the immunosuppressive domain were estimated based on homology to PERV-C (AM229312) (Preuss et al., 2006) and to other retroviruses (Fig. 1). The position of each transmembrane helix was determined using HMMTOP (Tusnady and Simon, 1998, 2001). Both FPPR (E1) and MPER (E2) overlap with one of the outside tails. Non-synonymous nucleotide mutations that were also detected in koala museum specimens (Tsangaras et al., 2014) are shown in boldface. The HR1 and HR2 motifs are shown as underlined amino acids, the Cl binding site as an underlined and bold amino acid, the amino acids comprising the CX(6)C motif are italicized, and the amino acids of the homotrimer interface are indicated using diamonds.

the p15E, which would alter the 13 amino acids downstream of the deletion and also change the stop codon at the end of p15E to code for a residue.

The 163 clonal sequences with intact open reading frames (ORFs) included 39 distinct haplotypes (Table 1). The most common haplotype was present in all 4 koalas, and comprised 106 of the 163 sequences (Table S1). Of the other 38 distinctive haplotypes, 15 had only synonymous differences when compared to the most common haplotype. The 16 haplotypes with identical predicted amino acid sequences represented 139 of 163 clonal sequences with intact ORFs (Table S1), and the inferred polypeptide coded by these sequences was designated P15E-1. The remaining 23 DNA sequences were distinct from each other, and each coded for a different polypeptide with 1 or 2 residues different from p15E-1 (Table 1, Fig. 2). Among these 23 amino acid sequences, 5 were identified in the northern koala and 18 were detected in just one of the 3 southern koalas. One of these polypeptides, p15E-2, appeared in two identical clonal sequences of koala Pci-128, but was not detected in other koalas. The other 22 haplotypes were each present in only 1 clonal sequence from just 1 koala (Table 1). We also compared the non-synonymous nucleotide mutations present in the 22 haplotypes to p15E-1 polymorphisms identified in next generation sequencing data that had been previously generated from 7 historical museum koalas (Tsangaras et al., 2014). Among the non-synonymous nucleotide substitutions present in just one of the clones generated by the current study, 8 were also verified as a polymorphism among the museum specimens, present at a frequency of 3% or greater within the corresponding reads of at least one museum koala (Table 1).

Relative to the reference polypeptide p15E-1, there were two amino acid differences predicted in polypeptides p15E-4, p15E-5, p15E-8, and p15E-20 while 19 predicted polypeptides (including p15E-2) differed from p15E-1 at a single amino acid (Fig. 2, Table 1). Among 24 non-synonymous substitutions (Table S2), only two were shared among different polypeptides: A145T (the first position represents the residue in p15E-1) in p15E-2, p15E-4, and p15E-8; and V182A in p15E-4 and p15E-18. The other 22 non-synonymous substitutions were present in only one of the haplotypes. Of the 27 non-synonymous variants detected relative to p15E-1, 14 had variation detected in more than one clone, or at the same position in multiple clones (Fig. 2), or also detected in the museum dataset. It remains possible that some of the remaining haplotypes may represent cloning or other errors, although cloning error is expected to be rare (10^{-4} to 2×10^{-5}) (Life Technologies Corp, personal communication).

Sequences of p15E reported by previous studies were also examined (GenBank: JQ244835 to JQ244839, AF151794, DQ174772, AB721500, NC021704, AB822553, AB823238, AB828004, and AB828005) (Avila-Arcos et al., 2013; Fiebig et al., 2006; Hanger et al., 2000; Shojima et al., 2013a; Xu et al., 2013). For the five koala museum

Table 1
KoRV p15E non-synonymous variation across four koalas.

Polypeptide Designation		*N*	Non-synonymous substitutions vs. p15E-1	Number of clones coding for the polypeptide				
				Northern Australia Pci-SN404	Stony Rises Pci-157	Brisbane Ranges Pci-128	Kangaroo Island Pci-182	Total
		Detected in more than one clone						
KoRV-A	p15E-1	16	–	37	30	32	40	139
KoRV-A	p15E-2	1	1	0	0	2	0	2
		Detected in only a single clone						
KoRV-A	p15E-3*	1	1	0	0	1	0	1
KoRV-A	p15E-4	1	2	0	0	1	0	1
KoRV-A	p15E-5*	1	2	0	0	1	0	1
KoRV-A	p15E-6	1	1	0	0	1	0	1
KoRV-A	p15E-7*	1	1	0	0	1	0	1
KoRV-A	p15E-8	1	2	0	0	1	0	1
KoRV-A	p15E-9*	1	1	0	0	1	0	1
KoRV-A	p15E-10*	1	1	0	1	0	0	1
KoRV-A	p15E-11*	1	1	0	1	0	0	1
KoRV-A	p15E-12	1	1	0	1	0	0	1
KoRV-A	p15E-13*	1	1	0	1	0	0	1
KoRV-A	p15E-14	1	1	0	1	0	0	1
KoRV-A	p15E-15	1	1	0	1	0	0	1
KoRV-A	p15E-16	1	1	0	1	0	0	1
KoRV-A	p15E-17	1	1	0	1	0	0	1
KoRV-A	p15E-18	1	1	0	0	0	1	1
KoRV-A	p15E-19	1	1	0	0	0	1	1
KoRV-A	p15E-20*	1	2	1	0	0	0	1
KoRV-A	p15E-21	1	1	1	0	0	0	1
KoRV-A	p15E-22	1	1	1	0	0	0	1
KoRV-A	p15E-23	1	1	1	0	0	0	1
KoRV-A	p15E-24	1	1	1	0	0	0	1
		From GenBank						
KoRV-A	DQ174772	1	0	0	0	0	0	
KoRV-B			2	0	0	0	0	0
KoRV-J	OJ-4 11-1		2	0	0	0	0	0
KoRV-J	OJ-4 11-4		10	0	0	0	0	0
Total		43		42	38	41	42	163

Pci-SN404 is a zoo koala from Queensland; the other three are wild koalas from the southern Australian populations listed. *N* is the number of distinct haplotypes that coded for the same polypeptide (i.e., only synonymous differences were present). The haplotypes that coded for polypeptides p15E-3 to -24 each were detected only in one clonal sequence; some may represent cloning errors. However, for those with an asterisk (*) the same nucleotide change was detected among museum specimen KoRV polymorphisms (Tsangaras et al. 2014), including both polymorphisms in p15E-20. Two GenBank sequences (AF151794, AB721500) code for the same polypeptide as p15E-1; only GenBank entries that coded for different polypeptides are listed. Each listed Genbank entry coded for a distinct polypeptide: KoRV-A is GenBank number DQ174772, KoRV-B is NC021704, KoRV-J OJ-4 11-4 is AB822553. KoRV-J OJ-4 clones 11-1, 2, and 5 (AB823238, AB828004, and AB828005, respectively) had identical sequences coding the same polypeptide, listed here as 11-1.

specimens collected 30 to 120 years ago, due to DNA degradation only short sequence reads had been generated, and it was not possible to determine the phase of SNPs that were far apart (Avila-Arcos et al., 2013). The nucleotide sequences of AF151794 (Hanger et al., 2000) and AB721500 (Shojima et al., 2013a) matched haplotype p15E-1H1, which was the most common DNA sequence among our clones. Of 8 entries in GenBank for p15E from modern koalas, 2 coded for the same amino acids as p15E-1. The sequence DQ174772 (Fiebig et al., 2006) coded for one amino acid difference. Fig. 2 and Table S3 show that when compared to p15E-1, there were 2 non-synonymous mutations in KoRV-B (GenBank accession NC021704) (Xu et al., 2013), 2 non-synonymous mutations in KoRV-J OJ-4 clone 11-1 (AB823238; identical in sequence to clones 11-2 and 11-5, corresponding to AB828004 and AB828005, respectively) (Shojima et al., 2013b), and 10 non-synonymous mutations in KoRV-J OJ-4 clone 11-4 (AB822553) (Shojima et al., 2013b). None of the non-synonymous mutations present in the GenBank entries was detected in the sequences generated for this study. Importantly, all of the KoRV variants in GenBank, including the KoRV-B and KoRV-J sequences, coded for amino acid residues identical to those of p15-E1 in epitope 1, epitope 2, and the immunosupprressive domain.

Protein structure modeling

We examined the effects of non-synonymous variation on the protein structure of p15E in recently published variants of KoRV reported to be exogenous (Shojima et al., 2013b; Xu et al., 2013). We did not find any major changes in these variants (Figs. 3 and 4, Table S3). We also examined the potential effects of non-synonymous variation among newly generated sequences on the protein structure of KoRV p15E, using the most common p15E-1 polypeptide as a reference for three-dimensional modeling of the protein and all structural comparisons (Fig. S1). Both E1 and E2 were highly conserved among the different KoRV amino acid variants except L33P (E1, p15E-23) and E130G (E2, p15E-16), each of which appeared in the currently generated sequences only once (Fig. 2, Table 1). The immunosuppressive domain, which is highly conserved across retroviruses, was also conserved among the KoRV p15E amino acid variants, with only two variants, each detected only once among the 163 sequences (in p15E-7 and p15E-10). The structural models revealed that although overall the structure was highly conserved there were several changes affecting atom conformations and local surface charge (Figs. S1). Specifically, four amino acid variants (A8T, S54F, C94Y, and A145T) exchanged a buried or partially buried amino acid for a surface residue. Six variants (E93G, E130G, L159P, V163A, R184G, and E193A), five of which were "radical" (defined as non-synonymous mutations with a negative score in both BLOSUM62 and BLOSUM90 substitution matrices), had the opposite effect. Thirteen amino acid variants, five of which were radical substitutions (S23P, L33P, L76P, F96S, and S101P), would not affect the localization of the R-group (Figs. 1, 2, and S1 and Table S2). Furthermore, several amino acid variations resulted in extensive local changes in the surface electrostatic potential of the protein structure (Table S2). Specifically, mutations A8T, A145T, K82E, N172D, and K82E would increase the local negative surface charge, while E93G, S101P, E193E, and N165D would increase the local positive charge (Fig. S1, Table S2). Additionally, six amino acid variants, S54F, L76P, K82E, E93G, C94Y, and F96S, five of which were radical (Fig. S1, Table S2), were found to change functional (as inferred by CDD) amino acids relative to p15E-1 (Fig. S2). These were located within ectodomain HR1 and HR2, two of them were at the immunosuppressive domain (L76P, K82E), and one (S54F) was part of the homotrimer interface.

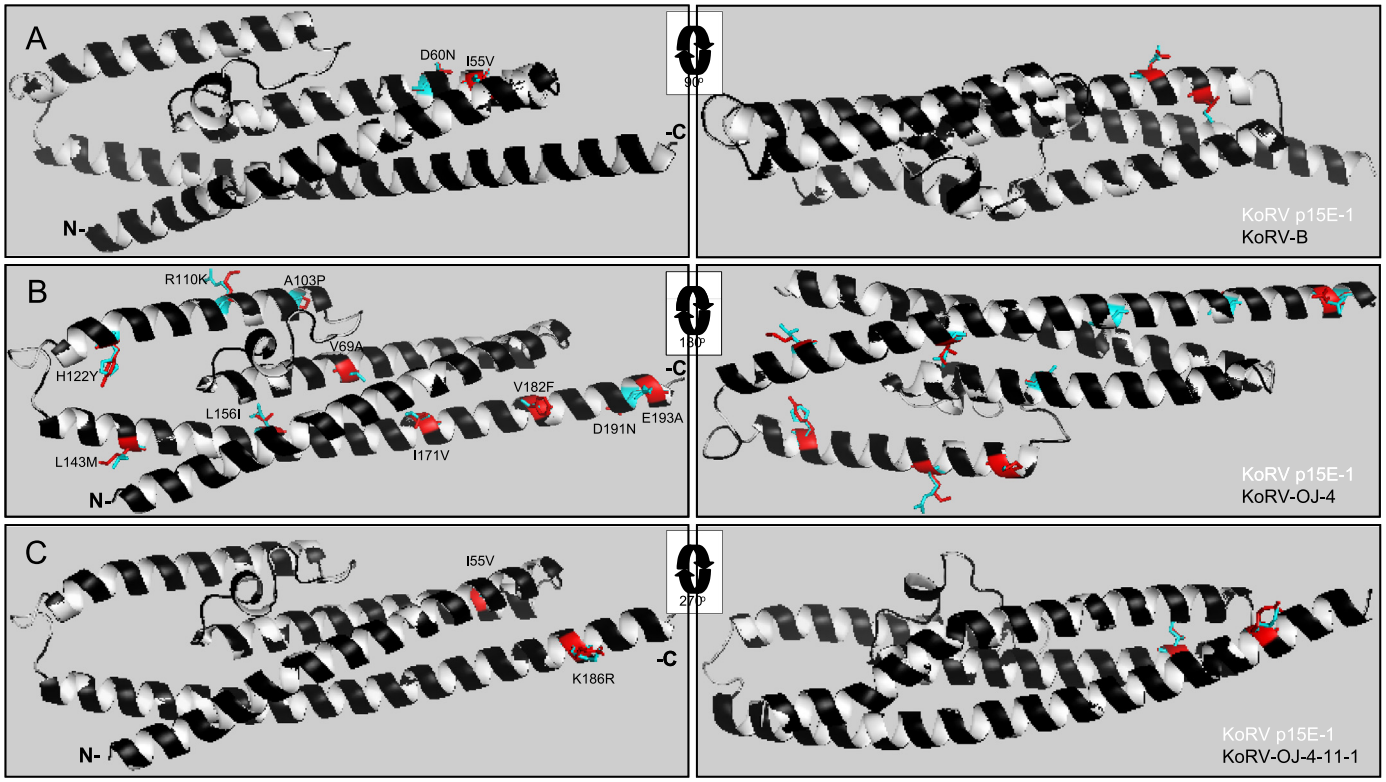

Fig. 3. Structural superimpositions between KoRV p15E-1 and KoRV variants reported as exogenous. The KoRV p15E-1 protein structure, which was used as a reference structure is shown in white and three other KoRVs reported to be exogenous (Shojima et al., 2013b; Xu et al., 2013) are shown in black. Shown are (top panels) a comparison between KoRV p15E-1 and KoRV-B; (middle panels) a comparison between KoRV p15E-1 and KoRV-J (OJ-4 clone 11-4); and (bottom panels) a comparison between KoRV p15E-1 and KoRV-J OJ-4 11-1 (OJ-4 clone 11-1; identical to clones 11-2 and 11-5). All comparisons demonstrate the overall similarity of the structures. Amino acid variations between sequences mapped on the protein models are shown. These are colored cyan for p15E-1 and red for all the other KoRVs. These variable amino acid residues are depicted in line representations to visualize the atoms. The models are presented in cartoon ribbon representations.

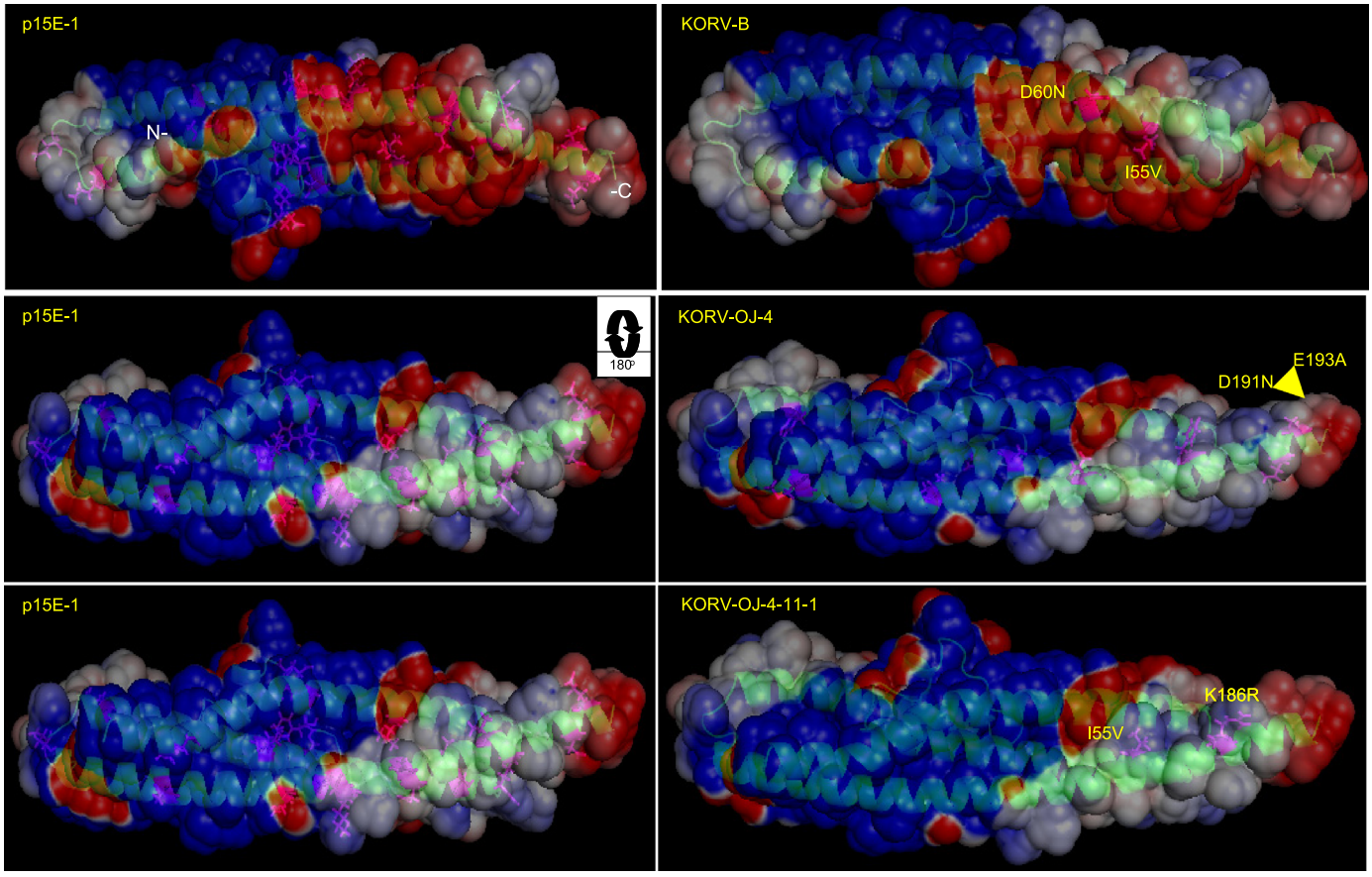

Fig. 4. The distribution of the electrostatic potential on the solvent-accessible surfaces of p15E is highly conserved. The electrostatic potential of KoRV p15E-1 (left panels) is compared to those of (right panels, top to bottom): KoRV-B, KoRV-J (OJ-4 clone 11-4) and KoRV-J OJ-4 11-1 (OJ-4 clone 11-1; identical to clones 11-2 and 11-5) (Shojima et al., 2013b; Xu et al., 2013). A notable difference due to mutations found in KoRV-J (OJ-4 11-4) sequence is shown with a yellow arrow (right middle panel). The regions colored in blue represent positively-charged groups, red represents negative charges, and white-gray areas are electrically neutral. Structures of all proteins are shown in equivalent orientations and are seen from two alternate views differing by 180°. The models are shown as semi-transparent surfaces to visualize the secondary elements and amino acid chains.

Signatures of selection

To examine whether p15E has been under selection, the 163 sequences with intact ORFs generated in this report were used to estimate Tajima's *D*, which is a test of neutrality (Tajima, 1989). If the value of Tajima's *D* is not significantly different from zero this provides support for neutrality. Tajima's *D* was −2.28 with 12 segregating sites and π (nucleotide diversity) of 0.0009. This strongly negative value for Tajima's *D* suggested that p15E has been under purifying selection. Strong evidence for purifying selection was also provided by other analyses of the coding region: a low *dN*/*dS* ratio (0.3) with $dN = 0.003 \pm 0.001$ (the number of nonsynonymous substitutions per nonsynonymous site) and $dS = 0.010 \pm 0.002$ (the number of synonymous substitutions per synonymous site); and the results of a codon-based *Z*-test of purifying selection, which averaged over all sequence pairs showed significant deviation from neutrality ($p = 0.002$, $Z = -2.92$, one-tailed test).

Discussion

The KoRV p15E protein contains an immunosuppressive domain that is highly conserved among different retroviruses (Denner et al., 1994). In HIV-1, single mutations in this domain have been shown to reduce IL-10 release, which implies that the immunosuppressive activity by HIV is abrogated by these mutations, although the impacts are different depending on the position of the mutation. Additionally, rats immunized with a mutated immunosuppressive domain of HIV-1 show higher antibody response (Morozov et al., 2012). Among HIV-1 infected patients, mutations that abrogate immunosuppressive activity are not common and it appears that viruses with a mutated immunosuppressive domain are selectively eliminated by the immune system, since the immunosuppressive domain suppresses antibody response in vivo (Morozov et al., 2012). Culturing KoRV with human peripheral blood mononuclear cells results in higher IL-10 production, which suggests that KoRV is immunosuppressive (Fiebig et al., 2006). The high conservation of the immunosuppressive domain across KoRV, GALV, and PERV (Fig. 1) suggests that p15E plays an important functional role in KoRV, as does our finding that p15E showed signatures of purifying selection. Furthermore, the p15E region contains the HR1 and HR2 motifs, the homotrimer interface; a chloride ion binding site; and the CX(6)C motif. We found that these domains are highly conserved across proviruses including KoRV variants reported to be exogenous: KoRV-B (Xu et al., 2013) and KoRV-J (Shojima et al., 2013b). Only a few mutations were predicted to affect protein structure, function, or antigenicity. A detailed study of PERV has shown that antibodies are directed only against epitope 2 (MPER) of p15E and not against epitope 1 (FPPR) (Waechter and Denner, 2014). We found one haplotype with an amino acid change in each of epitope 1 (p15E-23) and epitope 2 (p15E-16). Importantly, the variants in

these epitopes were not predicted to greatly influence the protein structure. These epitopes were also highly conserved in KoRVs reported to be exogenous: KoRV-B (Xu et al., 2013) and KoRV-J (Shojima et al., 2013b). This suggests the possibility that all KoRVs might be neutralized by vaccines developed using KoRV p15E.

The signature of purifying selection suggests that mutations in p15E had been deleterious for KoRV, and would explain why p15E remains conserved among different proviruses. The sequences on which these tests of selection were based may be derived from either exogenous or endogenous KoRVs. However, in a recent study of LTRs in endogenous KoRVs, it was determined that all of the detectable mutations present across endogenous KoRVs had occurred while the virus was still exogenous, and that not enough time had elapsed since endogenization for any mutations to have occurred across the ca. 10,000 bp of enKoRV sequences examined (Ishida et al., 2014). Thus the variation detected among KoRVs in our dataset is likely to reflect mutations and selection pressure that occurred only among exogenous KoRVs, even if these were detected among proviruses that had subsequently endogenized.

Despite a high degree of conservation and purifying selection, we found some mutations that may alter the structure and function of specific p15E domains, which are also likely to represent changes present before KoRV endogenized (Ishida et al., 2014). Protein modeling suggested that two radical substitutions in the immunosuppressive domain may affect the local protein conformation and alter the surface charge. These drastic changes could potentially alter the function of the highly conserved immunosuppressive domain. Because these variants were also detected in museum specimens, they are unlikely to represent cloning errors although each of the variants was detected by our study in only a single clonal sequence. There were four other variants in the trimer interface and the two viral ectodomains that affected amino acids of known function. These variants were predicted to alter the protein structure locally and may affect the trimerization, fusion, and infection of the viruses. Additionally, in some other cases the location of the R-group was altered and surface amino acids were exchanged for buried residues and vice versa. These changes could have important functional implications, because they may generate new or alleviate pre-existing antigenic sites, which may result in differences in immune response. Moreover, we found that certain amino acid variations (Fig. S2) were predicted to drastically change the local surface charge. Such changes in the KoRV p15E domain could have important biological implications, as has been shown for other viruses, where changes in charge surrounded pockets and electrostatic interactions with small molecules were found to affect trimer assembly, membrane fusion, and viral entry (Ramsdale et al., 1996; Zhu et al., 1998). Some of the variants were also identified in museum specimens (Fig. 2) suggesting that the variants persisted over time. Some variants, especially the ones that were not in frame, may represent proviruses that are not replication competent. Additionally some of the non-synonymous mutations may have occurred in endogenous KoRVs, after the proviruses integrated into the host germ line, in which case sequences with unusual mutations may not be expressed, and thus would have little or no relevance to the utility of a vaccine geared towards koalas unaffected by KoRV.

The transmembrane envelope protein p15E has been used in immunizing animals against gammaretroviruses (Denner et al., 2012; Kaulitz et al., 2011; Langhammer et al., 2011; Waechter et al., 2013). With the exception of FeLV in cats, studies of p15E as a vaccine candidate have not been conducted using the viral host species. Vaccines for FeLV currently rely on inactivated whole virus or on the non-glycosylated surface envelope protein, although neither type provides full protection, and p15E has been proposed as being of potential benefit for vaccination (Fiebig et al., 2003; Langhammer et al., 2006).

The current study found that p15E was highly conserved among KoRV proviruses in koalas from northern and southern Australia and in KoRV variants reported to be exogenous; that functional domains including the immunosuppressive domain and several epitopes were highly conserved between KoRV and similar retroviruses; and that none of the non-synonymous variation detected within KoRV in the two epitopes would greatly affect protein structure. KoRV p15E has been shown to induce neutralizing antibodies (Fiebig et al., 2006), suggesting that KoRV p15E may be a potential candidate for vaccine development. Of course, before widespread use, vaccine trials would be necessary to demonstrate their efficacy in providing protective immunity against KoRV in koalas. Vaccines would also have to be tested for safety, to ensure that they do not trigger unexpected side effects. These may be of particular concern in the case of KoRV since many koalas carry endogenous copies of KoRV that are present in every nucleated cell. In any future studies, it would be important to consider that the effects of any potential vaccine may vary depending on whether or not an individual koala carries endogenous copies of KoRV.

Materials and methods

Sample collection and DNA extraction

Ethical approval for this study was provided by the University of Illinois Institutional Animal Care and Use Committee, approved protocol number 12040. The koalas were chosen based on their known KoRV-positive status and their geographic diversity. They included three wild southern Australian koalas, one each from the Stony Rises (Pci-157) and the Brisbane Ranges (Pci-128) of Victoria, and one from Kangaroo Island (Pci-182) of South Australia. The southern koala samples had been collected in 1988 by the Laboratory of Genomic Diversity, National Cancer Institute, USA, under permit no. 87-150 issued by the Department of Conservation, Forests and Land, Victoria (Taylor et al., 1991). Their DNA had been extracted using a phenol-chloroform method at NCI, and sent to UIUC under Materials Transfer Agreement 2008-05798-01-00. One northern Australian (Queensland) zoo koala was also included, “Bunyip”, Pci-SN404 (“SN” represents the North American Regional Studbook number). The American Zoo Association's Species Survival Plan (AZA SSP) manages northern (Queensland) and southern koalas separately and the pedigrees are well characterized. The inbreeding coefficient of Pci-SN404 was estimated to be very low ($f \cong 0.008$). After the approval of the AZA koala SSP Management Group, the blood sample for Pci-SN404 was collected during a regular physical examination on September 2, 2010, at 4 years of age, with DNA extracted using the QIAamp DNA Blood Mini Kit from Qiagen (Valencia, CA).

PCR, cloning, and sequencing

The position of p15E in KoRV (GenBank: AF151794) (Hanger et al., 2000) was determined based on homology to gibbon ape leukemia virus (GALV; GenBank: NC_001885) (Delassus et al., 1989) and feline leukemia virus (FeLV; GenBank: NC_001940) (Chen et al., 1998). Primers for PCR were designed using the software Primer3 (http://fokker.wi.mit.edu/primer3/input.htm) (Rozen and Skaletsky, 2000) to target conserved DNA regions, as determined by an alignment of KoRV sequences DQ174772 (Fiebig et al., 2006) and AF151794 (Hanger et al., 2000), and to amplify the complete p15E region in a single amplification. Two alternative primer pairs were designed: p15E-F1 (CAGACGGTACCTTGCTACAGG) with p15E-R1 (CCTTCATTCCCCCATTTTCT) for an amplicon size of 693 bp; and p15E-F2 (GCTTGTCCCTCGCATCTACT) with

p15E-R2 (TTTTCTTTGAGGGTAGCTCTAATCA) for an amplicon size of 705 bp. For every koala, separate PCRs were run using each of the primer pairs.

PCR mixes included final concentrations of 0.4 μM of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTP (Life Technologies Corp., Carlsbad, CA), 0.8 μg/μl of bovine serum albumin (BSA; New England BioLabs Inc., Ipswich, MA) and 0.04 units/μl of AmpliTaq DNA Polymerase (Life Technologies Corp.). The PCR algorithm consisted of an initial denaturation at 95 °C for 5 min; followed by cycles of denaturation for 20 s at 94 °C, annealing for 3 cycles of 30 s at 60 °C, decreasing in subsequent cycles to 58 °C, 56 °C, 54 °C, 52 °C (5 cycles each) or 50 °C (final 22 cycles), and a 1 min extension at 72 °C; with a final extension of 7 min at 72 °C. An aliquot of each PCR product was examined on a 1% ethidium bromide stained agarose gel under ultraviolet light. PCR amplicons were treated with Exonuclease I (USB Corporation, Cleveland, OH) and shrimp alkaline phosphate (USB Corporation) to remove excess primers and unincorporated dNTPs (Hanke and Wink, 1994). Sanger sequencing was conducted as previously described (Ishida et al., 2011) and used the ABI 3730XL capillary sequencer at the UIUC Core DNA Sequencing Facility. Sequencher 5.1 (Gene Codes Corp.) was used to examine, edit and concatenate the sequences.

After Sanger sequencing verified that primers had amplified the target region, PCR products were cloned using a TOPO TA Cloning kit (Life Technologies Corp.) following the manufacturer's instructions. We examined the number of clones that would with 95% confidence identify minor alleles and haplotypes present at a frequency of 2.5% or more, and that would also identify a large majority of those present at 1% frequency. Forty-eight colonies from each koala sample were directly PCR-amplified following the protocol above (but without the use of BSA). Amplicons were examined on a gel, purified, and Sanger-sequenced following the procedures described above. The sequences were edited, trimmed to include only the p15E coding region and aligned using the software Sequencher 5.1 (Gene Codes Corp.). All distinct sequences that coded p15E-1 to p15E-24 were deposited in GenBank, under accession numbers KJ764672–KJ764710. Non-synonymous polymorphisms that appeared only once among the clones were suspected of being cloning artifacts; to minimize this possibility, these polymorphisms were searched for among the polymorphisms that had been previously detected among samples of 7 museum koalas (Tsangaras et al., 2014).

Identification of protein domains and functional residues, and protein modeling

MEGA 5.2 (Tamura et al., 2011) was used to translate and align the amino acid sequences. The positions of KoRV epitope 1 (E1), epitope 2 (E2), and the immunosuppressive domain were inferred based on homology to the corresponding regions in pig endogenous retrovirus (PERV) (Fiebig et al., 2003) and other retroviruses (Denner et al., 1994). Transmembrane helices were identified using the software HMMTOP (http://www.enzim.hu/hmmtop) (Tusnady and Simon, 1998, 2001). Domains in the amino acid sequences were identified using the Conserved Domains Database (CDD) of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (Marchler-Bauer et al., 2013). The Conserved Features/Sites option of the CDD was used to examine whether non-synonymous mutations were present within protein regions of known function. Those non-synonymous mutations that produced a negative score in both BLOSUM62 and BLOSUM90 substitution matrices were defined as "radical."

To determine the structural characteristics of KoRV protein variants, their three-dimensional (3D) structures were predicted. The p15E-1 sequence was used as a reference and its 3D structure was predicted using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Roy et al., 2010; Roy et al., 2012; Zhang, 2008). I-TASSER uses a combination of alignments, threading, simulations, and structural assemblies to generate a final model (Roy et al., 2010; Roy et al., 2012; Zhang, 2008). Because the structure of KoRV has not been solved experimentally, to model KoRV p15E, a combination of different structural data was used from several retroviral proteins: 2xz3A, bovine leukemia virus; 1eboA, Ebola virus; 4g2kA, Marburg virus; 1y4mA, human *syncytin*-2 (endogenous retrovirus); 1mofA, Moloney murine leukemia virus (MoMuLV); and 3s88J, Sudan Ebola virus. These structures were utilized to generate the model because they had the highest percentage of identity to KoRV p15E sequence and they produced high confidence threading alignments (Roy et al., 2010; Roy et al., 2012; Zhang, 2008). Then each of the variant proteins was modeled using the p15E-1 structure as a reference using the SWISS-MODEL server (http://swissmodel.expasy.org/) (Arnold et al., 2006). Using this strategy the whole p15E region could be reliably modeled. Pairwise structural alignments and structural superimpositions were performed using the DaliLite server (http://www.ebi.ac.uk/Tools/structure/dalilite/) (Holm and Park, 2000). Models and figures were drawn using Pymol (DeLano Scientific).

Tests of selection

MEGA, version 5.2 (Tamura et al., 2011) was used to implement the Nei-Gojobori method of determining the proportion of synonymous substitutions per synonymous sites and the proportion of non-synonymous substitutions per non-synonymous sites; to estimate Tajima's D ($D=0$: neutral, $D<0$: purifying selection, $D>0$: balancing selection); and to implement the codon-based Z-test of purifying selection using bootstrapping (1000 replicates).

Acknowledgments

The project described was supported by Grant number R01GM092706 from the National Institute of General Medical Sciences (NIGMS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIGMS or the National Institutes of Health. NN was supported by start-up funds and a state-mini grant from California State University, Fullerton. CM was supported by an HHMI scholarship. KMH was supported by the Smithsonian Institution. For modern koala samples, we thank M. Malasky, R. Hanson, S. O'Brien, M. Bush, J. Graves, W. Sherwin, N. Murray, and D. Wildt; and the Columbus Zoo and San Diego Zoo.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.10.036.

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