Research Article

Characterization of Porcine Endogenous Retrovirus Clones from the NIH Miniature Pig BAC Library

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Received 27 April 2011; Revised 8 June 2011; Accepted 16 June 2011

Academic Editor: Guihua H. Bai

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Pigs have been considered as donors for xenotransplantation in the replacement of human organs and tissues. However, porcine endogenous retroviruses (PERVs) might transmit new infectious disease to humans during xenotransplantation. To investigate PERV integration sites, 45 PERV-positive BAC clones, including 12 PERV-A, 16 PERV-B, and 17 PERV-C clones, were identified from the NIH miniature pig BAC library. The analysis of 12 selected full-length sequences of PERVs, including the long terminal repeat (LTR) region, identified the expected of open reading frame length, an indicative of active PERV, in all five PERV-C clones and one of the four PERV-B clones. Premature stop codons were observed in only three PERV-A clones. Also, eleven PERV integration sites were mapped using a 5000-rad IMPRH panel. The map locations of PERV-C clones have not been reported before, thus they are novel PERV clones identified in this study. The results could provide basic information for the elimination of site-specific PERVs in selection of pigs for xenotransplantation.

1. Introduction

Xenotransplantation is the transplantation of living cells, tissues, and organs from one species to another. Xenotransplantation can be beneficial in overcoming the shortage of human organs and tissues for allotransplantation. Pigs are considered as the best xenotransplant organ donor due to anatomical and physiological similarities with humans, and the relatively less financial and ethical problems compared with primates. However, the use of porcine samples for human xenotransplantation suffers from possible infectious risks due to the transmission of porcine endogenous retroviruses (PERVs) from pigs to humans [1, 2]. A recent study reported that PERVs can be transmitted to human cells via supernatants of primary porcine liver cells for short term using a bioartificial liver (BAL) model [3]. Despite this report, more than 200 human patients who received pig cells or tissues did not display PERV transmission in the

blood cells [4]. Another study reported the absence of PERV transmission from 21 patients receiving porcine islets and Sertoli cells for 4.6–8 years [5]. Conversely, another study suggested that PERVs and human endogenous retroviruses (HERVs) could possibly form new viruses by recombination [4], complicating the transmission of PERV infection via xenotransplantation.

PERVs are present in the genome of all pig breeds and are classified as three main types: PERV-A, PERV-B and PERV-C. PERV-A and PERV-B can infect human cells as polytropic viruses and PERV-C infects only porcine cells as an ecotropic virus [2]. A very recent study suggested that recombinant PERV-A and PERV-C might be important infectious risk factor in human cells [6]. Therefore, eliminating infectious PERVs is a crucial issue in xenotransplantation. For knock-out of active PERVs in the porcine genome, accurate PERV integration sites have to be identified. However, PERV copy numbers differ among pig breeds [7, 8], and there are

variations in PERV integration sites among breeds [9, 10]. Recent success in the knockdown of PERV expressions has been reported using small interfering RNA (siRNA) [11, 12]. For these biomedical experiments, miniature pigs are widely used mainly because they have the similar organ sizes to human [13, 14].

Long terminal repeats (LTRs) in the virus are mainly composed of the U3, R, and U5 regions. LTRs have important roles for the integration, replication, and regulation of retrovirus expression. Sequences in the R and U5 regions can repress PERV transcription [15]. Moreover, the level of PERV expression is associated with copy number of a 39 bp repeat in the PERV U3 region, which has an LTR transcription factor binding site [16]. A recent analysis of PERV LTR structures using sequences derived from public database of pig genome identified structural differences in the U3 region [17].

To characterize PERVs in National Institutes of Health (NIH) miniature pigs, the NIH miniature pig Bacterial Artificial Chromosome (BAC) library was screened and BAC end-sequences were used for the construction of contig maps for PERV-positive BAC clones and investigation of PERV map locations in the pig genome. Also, full-length PERV sequences, along with LTR sequences, were presently obtained. These results should provide basic information for selection of pigs for xenotransplantation research.

2. Materials and Methods

2.1. BAC Library Screening. A NIH miniature pig BAC library of approximately 15,000 clones was screened for PERV-positive clones by PCR analysis using three envelop-specific primers. PERV-specific primers were used for the identification of the PERV-A, PERV-B, and PERV-C BAC clones (Table 1). Polymerase chain reaction (PCR) was performed for PERV type identification with 20 ng template DNA, 1X PCR buffer, 2 mM MgCl₂, 10 mM of each dNTP, 0.2 μM of each primer and one unit of *Taq* polymerase (GeNet Bio, Republic of Korea). The thermal profiles included an initiation denaturation at 94°C for 5 min, following 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for env-A and -B and 56°C for env-C for 30 sec and extension at 72°C for 30 sec and then a final extension step at 72°C for 5 min using a PTC-200 Programmable Thermal Controller (MJ Research, USA). PCR products were analyzed on 2% standard Tris/Acetate/EDTA (TAE) agarose gels stained with ethidium bromide (EtBr).

2.2. BAC End-Sequencing and Construction of Contig Maps. The 5' and 3' BAC end-sequences (BESs) were obtained from 45 PERV-positive BAC clones using T7 and SP6 universal primers for the cycle sequencing reaction. PCR products were run on a model 3730XL automated DNA sequencer (Applied Biosystems, USA). After masking of the repetitive sequences in the BESs using RepeatMasker software (<http://www.repeatmasker.org/>), the sequences were subjected to BLAST searches against NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST>). BAC contig maps

were constructed using the primers designed from BESs with the PRIMER 3 software (<http://frodo.wi.mit.edu/primer3/>) (Table 2). PCR mixtures contained 20 ng of template DNA, 1X PCR buffer, 2 mM MgCl₂, 10 mM of each dNTP, 0.2 μM of each primer, and one unit of *Taq* polymerase (GeNet Bio, Republic of Korea). The PCR amplification was carried out in a PTC-200 Programmable Thermal Controller (MJ Research, USA) for 25 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec and then a final extension step at 72°C for 5 min. PCR products were separated using the 2% standard TAE agarose gels stained with EtBr.

2.3. Deduced PERV Amino Acid Sequences and Analysis of LTR Region. The identified positive BAC clones were validated by sequencing to determine whether the BAC clones possessed full length of PERV including the gag, pol, and env genes. To ascertain whether PERV sequences could make correct virus proteins, the amino acid sequences of each gene were deduced from nucleotide sequences using the open reading frame finder (ORFfinder) at NCBI (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). To characterize the LTR of each clone, nucleotide sequences of the LTR were aligned using ClustalW program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) [21]. Repeat sequences in the LTR region were investigated using a tandem repeat finder [22].

2.4. PERV Mapping with IMpRH Panel. Chromosomal locations of PERV-positive clones were identified using the INRA/University Minnesota porcine Radiation Hybrid (IMpRH) panel [23, 24] using primers originating from BESs in the PERV-positive BAC clones (Table 2). PCR conditions were initiation heating at 94°C for 30 sec; 35 cycle of at 94°C for 30 sec, at optimal annealing temperature of each primer set for 30 sec, at 72°C for 30 sec; a final elongation at 72°C for 5 min using a PTC-200 Programmable Thermal Controller (MJ Research). The PCR mixture included 25 ng templates, 1X PCR buffer, 2 mM MgCl₂, 10 mM of each dNTP, 0.2 μM of each primer, and one unit of *Taq* polymerase (GeNet Bio). The PCR products were sequenced to confirm whether correct target PCR products were amplified. The chromosomal assignments were determined using the IMpRH web server (<http://imprh.toulouse.inra.fr>).

3. Results and Discussion

3.1. Identification of PERV-Positive BAC Clones and BAC Contig Maps. Three types of PERV-positive clones were identified by PCR using three envelope-specific primers. From the 15,000 BAC clones in the library, forty-five PERV positive BAC clones were identified including 12 PERV-A, 16 PERV-B, and 17 PERV-C clones. In contrast to previous results that PERV-A and -B exist in the genomes of all pig breeds [25], the present study confirmed that the PERV-C could be found in only a few pig breeds [26], which is different from PERV-A and PERV-B that were reported in all multitransgenic pigs generated for xenotransplantation. Another study documented high PERV-C copy numbers in

TABLE 1: Type-specific primers for screening PERV-positive clones from NIH miniature pig BAC library.

Gene	Primer sequence (5' → 3')	Annealing temperature (°C)	PCR product (bp)
Env-A	F: TCCGTGCTTACGGGTTTTAC R: TTGCCAATCTTTCCATCTCC	60	224
Env-B	F: TAAAAGCACACCTCCCAACC R: CCGGAATTGACAAAGGAGAA	60	192
Env-C	F: CACCTATACCAGCTCTGGAC R: GTTAGAGGATGGTCCTGGTC	56	310

the genome of the miniature pig line [25]. The present study also identified a relatively high percentage of PERV-C clones (17/45; 38%) among 45 PERV-positive BAC clones.

To construct BAC contig maps, 5' and 3' BAC ends from 45 PERV-positive BAC clones were sequenced and primers for contig mapping were designed from the 90 BESs derived from both T7 and SP6 ends (Table 2). Of these, BLAST searches against the NCBI database indicated that nine clones (2 PERV-A, 1 PERV-B and 6 PERV-C clones) were already present in the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>). Before designing primers for the contig mapping, repetitive sequences were screened using RepeatMasker software (<http://repeatmasker.org>). Fifty-two BESs derived from 26 T7 primer ends and 26 SP6 primer ends were successfully used to construct contig maps of PERV-positive BAC clones. Sixteen PERV BAC clones (seven PERV-A clones (A1-1F, A1-1E, A1-2G, A4-1H, A1-6E, A1-6C, and A4-1G); four PERV-B clones (B3-7F, B4-13H, B1-8D, and B3-7G); five PERV-C clones (C1-1D, C4-2G, C1-10G, C3-1E, and C3-4B)) were mapped using both T7 and SP6 end-sequences. However, 10 PERV-positive BAC clones (1 PERV-A clone (A4-14H); 5 PERV-B clones (B1-7A, B3-11C, B3-3G, B4-1B and B3-12H); 4 PERV-C clones (C3-6F, C2-5F, C1-9B, and C1-9D)) were only mapped with T7 ends. Also, 10 PERV-positive BAC clones (2 PERV-A clones (A3-9F and A3-10E); five PERV-B clones (B2-10D, B2-11A, B1-11C, B4-2E, and B3-12E); 3 PERV-C clones (C2-6H, C2-3F, and C2-6C)) were only mapped with SP6 ends. Especially, one PERV-A clone (A4-1G), one PERV-B clone (B3-7G) and three PERV-C clones (C1-10G, C3-1E, and C3-48) did not overlap with other contigs and so were designated as singletons. On the other hand, nine PERV-positive BAC clones (two PERV-A clones (A3-7A and A3-5B); two PERV-B clones (B1-11F and B1-11G); five PERV-C clones (C1-12A, C1-12C, C1-2D, C1-5F, and C3-5G)) could not be mapped with both T7 and SP6 ends. The results indicated that 42.2% (38/90) of the BESs contained repetitive sequences and could not be used to design primers for construction of contig maps due to the presence of large portion of repetitive sequences in the BESs (Figure 1).

3.2. PERV Amino Acid Sequences and Analysis of LTR Region.

Because PERVs can be transmitted in a Mendelian fashion from parent to offspring through multiple generations, a large number of PERVs are known to be inactive. Therefore, deducing the amino acid sequence of a PERV is important to identify possibly active PERVs. Full-length PERV nucleotide sequences were obtained from 12 selected positive BAC

clones containing three PERV-A (A1-1F, A1-6C, and A4-1G), four PERV-B (B3-7F, B3-3G, B3-7G, and B3-12E) and five PERV-C (C1-9B, C4-2G, C1-10G, C2-6C, and C3-1E). The nucleotide sequences were deposited in the GenBank database (Accession numbers HQ536005-HQ536016). All three selected PERV-A clones had low possibility to be active viruses because they had nonsense mutations in the envelope protein. However, one clone (B3-7F) from four PERV-B BAC clones and five PERV-C BAC clones had an intact open reading frame (ORF) and so a high possibility of the production of virus particles, indicating that they were active PERVs (Figure 2). Even though PERV-C alone could not infect human cells, the PERV-A and PERV-C recombinant types can increase infectious risk in relation to xenotransplantation [27]. Therefore, identification of pig lines free of PERV-C is also very important.

LTR sequences from 12 selected PERV-positive BAC clones were characterized. The identified sequence homologies in the LTR R and U5 regions were 96.5%–100%. The possible reason for this high sequence conservation is that these two regions had TATA signal and cap sites related with transcriptional initiation. However, a previous study reported variable repeat numbers and differences in length of the U3 region [17]. Presently, similar results were obtained for the differences in repeat sequences in U3 regions (Figure 2). Three PERV-A LTRs were investigated and the sequence results revealed similar repeat patterns. Analyses of four PERV-B LTRs indicated that one (clone ID: B3-7F) displayed differences in repeat numbers. One of the PERV-C LTRs (clone ID: C3-1E) had an insertion in the U3 region and another PERV-C LTR (clone ID: C1-10G) had a 98 bp tandem repeat sequence in the U3 region. Recent studies associated the expression activity of PERV with the methylation status of LTRs [28, 29]. Also, LTR elements might be used for the prediction of transcription activity as well as evolution because of insertions and deletions in the LTR region due to recombination event among different PERV types [17]. Two PERV-C clones (clone ID: C1-10G and C3-1E) had recombinant PERVs in the LTR region; further research should be carried out with these recombinant PERVs, especially when the pigs are used for the xenotransplantation research studies.

3.3. PERV Integration Sites. To investigate PERV map locations in the porcine genome, a linker-mediated PCR method was applied. Previously, PERV chromosomal integration sites were characterized in three pig breeds including Large White,

TABLE 2: PCR conditions and primer information for contig mapping of PERV positive clones.

Type of PERV	BAC clone ID	Primer sequence (5' → 3')		Annealing temperature (°C)	PCR product (bp)	
A	A1-1F	T7	F: CCTGAGCGTGAAAAGATTCC	R: GGCCACTTGATTATGGCAGT	56	298
		SP6	F: CCATGTAGAAAGAGACACCTGTTG	R: TGAAGGTCCTGAGGTGTATGG	56	290
	A1-1E	T7	F: CCTGAGCGTGAAAAGATTCC	R: GGCCACTTGATTATGGCAGT	56	298
		SP6	F: CCATGTAGAAAGAGACACCTGTTG	R: TGAAGGTCCTGAGGTGTATGG	56	290
	A1-2G	T7	F: CCTGAGCGTGAAAAGATTCC	R: GGCCACTTGATTATGGCAGT	56	298
		SP6	F: CCATGTAGAAAGAGACACCTGTTG	R: TGAAGGTCCTGAGGTGTATGG	56	290
	A1-6C	T7	F: CCAATGATTCACTGACCTTTGA	R: TCCCTAGAGGGCTTCCCTTC	56	214
		SP6	F: ACGTGGAGACGTGAAGAACC	R: GACTCGAATCCAGCCTAAACC	56	111
	A1-6E	T7	F: TAGCATTTTCCATCCCAAACC	R: TTGCAGAGCAGATACTGACTCAC	56	105
		SP6	F: AGGTGGAAGCTACCTCAGCA	R: GCAGAGCTCTTACGCAGTCA	63	186
	A3-9F	SP6	F: TCATGTGCAGTTCAATGGAAG	R: TGGGAGTGGAGATTGTCCCT	56	183
	A3-10E	SP6	F: TCATGTGCAGTTCAATGGAAG	R: TGGGAGTGGAGATTGTCCCT	56	183
	A4-1G	T7	F: CTACGAAGGGTCTGAGCAC	R: TGGGCAGGTATTGAGGTTTC	56	174
	A4-1H	SP6	F: CTGTCAGTGTGAAGGGCTGA	R: CTCTGGCTTCTTCCCTCTT	63	248
T7		F: CATTGTTTGTCCAGTGTGTG	R: ATACCTGGCTTGGCACACAG	56	163	
SP6		F: AGGCTCATTTTTCATGCCAAT	R: GCCACATTTTGTTCATATGG	56	247	
T7		F: TGAAGACCAATGTCTGTGG	R: TGCCTTCAATTAAAGCCCTCA	63	229	
B1-7A		T7	F: TCAAAAGTCCTCCCAGTGTT	R: CATTGACTAGGGCAGGGGTA	56	180
		T7	F: AGCTCACCAGGAAAGGCATA	R: TCTCCAGTATCCTGCGTTTG	56	111
B	B1-8D	SP6	F: AATTCTCCCCTGGTTTCACC	R: TGCAGTTGGAGGGAGCTAAT	56	126
	B1-11C	T7	F: TTGGGTGTATTTTGTGGCTTC	R: GCAACCAGTTCAAGGCACAG	56	282
	B2-10D	SP6	F: GTTTCGTAGGGCCAAAACAGC	R: GGCACCCCTAGAGTTTTCAG	56	154
	B2-11A	SP6	F: GTTTCGTAGGGCCAAAACAGC	R: GGCACCCCTAGAGTTTTCAG	56	154
	B3-3G	T7	F: AAAGCTCTATCCTTTGGAGACAGA	R: TACTGGTGGCATGTTTGA	56	211

TABLE 2: Continued.

Type of PERV	BAC clone ID	Primer sequence (5' → 3')		Annealing temperature (°C)	PCR product (bp)		
B	B3-7F	T7	F: CAGCCGTTGCAGAAATCTTA	R: GCAAGGTAAGCCAAAGGATTG	60	182	
		SP6	F: CTGCAGGGGAAAATAGATGGTT	R: GACCATGGAGAGAAAGGTTG	56	259	
	B3-7G	T7	F: TCCAAAGCTTAGAAGGATGCAA	R: GCTCATGCTAGCAAAAGCCTTA	60	244	
		SP6	F: GAGCAACGTGCTAAACAAAAC TG	R: TTTGTTATGACAATGTGGTTTCA	56	150	
	B3-11C	T7	F: CCACAGCTAACGGAGTGTT	R: AAAGAAAACAGGCAGGGGTTT	56	204	
	B3-12E	SP6	F: TGTGCCCAACTGCTACTGTC	R: TACCCTCATGGGCAATCTC	60	263	
	B3-12H	T7	F: CTGCAACAGAATGGCATAAGA	R: GCACTCGATGTGCTTTTCA	60	138	
	B4-1B	T7	F: CCCACTGGAAAAAGGAAAGA	R: ATTAGCGGGTGCCCTTCTAT	56	199	
	B4-2E	SP6	F: ACTCCCAGGGAATTCAGGTT	R: TGTGTTATTCAAAGGCAGGAA	56	158	
	B4-13H	T7	F: GTTTC TGAGGCCAAAACAGC	R: GCCACCCCTAGAGTTTTCAG	56	156	
		SP6	F: AGAGAAAGGAAGGGGAGGACA	R: CTGCTTCTCTCCAGTCTCAG	56	108	
	C	C1-1D	T7	F: TCTAGGCCCAATTGGTTTGG	R: TCACCTTAGCCCCGACCCCATAG	63	200
			SP6	F: TCATCTAAATGCTTGTGTTTG	R: AAATGCCCTGTGCCCTTTTGAG	63	281
C1-9B		T7	F: ATGGAGCTGACAAACCCCTG	R: CAATCACAAAGCCACATGTCC	63	197	
C1-9D		T7	F: ATGGAGCTGACAAACCCCTG	R: CAATCACAAAGCCACATGTCC	63	197	
C1-10G		T7	F: CTCTGGTCTGGGCAAAATAC	R: CAGCCTTTCCATCACTCCAC	63	214	
		SP6	F: TGTATCCAGGAGGCATCACA	R: TTGGTCTGCATTGAGGACTG	63	194	
C2-3F		SP6	F: CAGCAGTGGTCTGTTGAGA	R: CCAGCCTTTGTCACTCTTCAC	63	265	
C2-5F		T7	F: TTTTCGTTGCTCTACACTTGA	R: GGTGGAGTGGAGTTTGTGGA	63	205	
C2-6C		SP6	F: TGCAATAGAGCAAAAGGGAGAA	R: AACCTGACCTTCCCCAAAGAT	63	268	
C2-6H		SP6	F: GGAAGATTTTGGATACGTGTG	R: TGAGTTTTCACAGTTGCATGG	60	163	
C3-1E		T7	F: CTCTGAGGACACAGGCTTCC	R: ACTGCCACTCCAACCACTCT	60	150	
		SP6	F: CAGAACCCGTCCACTGAAAT	R: TCCAGGGAGAAAACCTGGTTG	63	196	
C3-4B		T7	F: CTCTGAGGACACAGGCTTCC	R: ACTGCCACTCCAACCACTCT	60	150	
		SP6	F: CAGAACCCGTCCACTGAAAT	R: TCCAGGGAGAAAACCTGGTTG	63	196	
C3-6F		T7	F: CGTGTTT TGAGGAGCACACAT	R: GTCCACAGAAAGCCAGAGAGG	63	211	
C4-2G		T7	F: ATAATCACAATCTGTGGGCATA	R: GCTCAACCCCGAGACATTTAGAC	60	108	
		SP6	F: TGCTGTTCTTTACTGGGCATT	R: ATCCCAGCTGCCAAATACAC	63	265

FIGURE 1: Contig maps for PERV-positive BAC clones using primers designed from BAC end-sequences. Fifteen PERV containing contig maps were constructed in this study ((a)–(d)) PERV-A type; ((e)–(h)) PERV-B type; ((i)–(o)) PERV-C type and five contigs ((c), (g), (l), (n) and (o)) were identified as singletons. Solid circles indicate that primers were designed from the BAC end-sequences and open circles indicate that no primers were designed from the BAC end-sequences due to the repetitive sequences. Also, the presence of common BAC end-sequences among the PERV-positive BAC clones is indicated by cross mark.

FIGURE 2: The organization and structure of PERV LTRs identified from the NIH miniature pig BAC clones (a) and the different subrepeat sequences identified in PERV LTRs (b).

TABLE 3: Comparison of characterized PERV-A, PERV-B and PERV-C integration sites in different four breeds.

Pig chromosome	NIH miniature pig			Korean native pig ¹		Large White pig ²		Westran pig ³	
	A	B	C	A	B	A	B	A	B
1						1q2.1 1q2.3 1q2.4		1q1.2	1p2.2
2	2q1.3-q2.1				2q2.1 or 2q2.2		2q2.1	2p1.4	
3		3q2.1-q2.7 3q1.1-q1.4						3p1.4	
4		4					4p1.1		
5								5p1.3 5p 1.2 5q 1.2 5q2.1	5q1.2
6	6q3.5							6p1.4 6q3.5	
7				7p1.3	7p1.1		7p1.1 7p1.2-p1.1	7p1.3 7q1.5	7p1.2
8						8p1.2			8p2.2
9					9q2.6		9q2.6	9q2.4	9q2.4
10							10p1.2		
11					11q1.1 or 11q1.2		11q1.2 11q1.4		11p1.3
12			12p1.5					12p1.3	12q1.1
13	13		13 13q4.1-q4.9	13q4.2 or 13q4.3		13q4.2 13q4.3 13q4.9	13q4.2	13q4.1	13q4.1
14							14q2.8		14q1.3
15			15						
16								16q2.1	16q2.1
17				17q1.2	17q2.1		17q2.1	17q1.4	17q2.1
18		18q2.4							
X								Xp2.1	Xp1.3
Y						Yp1.2		Yp1.1	Yq

¹ Published locations in Korean native pig [18].² Published locations in Large White pig [19, 20].³ Published locations in Westran pig [10].

Westran, and Korean native pigs [10, 18–20]. In this research, 11 PERV genomic locations, consisting of three PERV-A, four PERV-B, and four PERV-C clones, were identified in NIH miniature pigs and compared with previously published integration sites in other three breeds. Eleven PERV-positive BAC clones were randomly selected from contig maps in each of the loci and the chromosomal locations have been determined using 5000-rad Radiation Hybrid panel [24] with primers designed from the BESs (Table 2). Of these, three map locations on SSC2, SSC6, and SSC13 were identified for the PERV-A clones. The PERV-positive BAC clone A4-14H, located on SSC6q3.5 and significantly linked with SW2419 marker (LOD score = 14.72), corresponded to a site previously reported in the Westran pig. The fluorescence in situ hybridization (FISH) mapping technique used for the

PERV mapping in Westran pigs indicated that they are more possibly the same PERVs. Also, the PERV BAC clone A1-F1, located on SSC2q1.3-q2.1 and importantly linked with the SWR1342 marker (LOD score = 19.36), represented a unique integration site not found in other three breeds. Also, the PERV BAC clone A1-6C mapped to SSC13 linked with the SW955 marker (LOD score = 4.18). Four PERV-B clones were mapped on SSC3, 4 and 18. Two PERV-positive BAC clones, B3-7G located on SSC3q1.1-q1.4 and B3-12E located on SSC3q2.1-q2.7, were highly linked with the SW1045 (LOD score = 8.66) and SW717 (LOD score = 18.47) markers, respectively. Also, the PERV-positive BAC clone B3-7F was mapped on SSC 18q2.6 linked with the S0062 marker (LOD score = 5.64). Three PERV-B locations identified in NIH miniature pigs turned out to possess unique integration sites

that have not been identified previously. Also, another PERV BAC clone, B3-3G, has been mapped on SSC4, which was linked to SW1003 marker (LOD score = 3.18). According to previously reported integration sites, PERV-C types were not observed in Large White, Westran, and Korean native pigs. However, PERV-C clones were mapped in four chromosomal locations in the NIH miniature pigs. Two PERV-positive BAC clones, C1-10G located on SSC12p1.5 and C2-6C located on SSC13q4.1-q4.9, were significantly linked with the SW2490 (LOD score = 13.44) and SW769 (LOD score = 8.31) markers, respectively. The other two PERV positive BAC clones, C3-6F located on SSC15 and C4-2G located on SSC13, were linked with SW1892 (LOD score = 6.64) and S0084 (LOD score = 7.81), respectively. These PERV-C-positive clones were not mapped previously; the present study is novel in identifying their map locations. In summary, only one PERV-A location on the SSC 6q3.5 (clone ID: A4-14H) in the NIH miniature pig correspond to integration site previously identified in the Westran pig, and six distinct retroviral integration sites were found on SSC 2, 3, 12, 13, and 18. On the remaining pig chromosomes, including X and Y, any specific PERV genomic sites were not found in this study (Table 3).

In this study, 45 PERV-positive BAC clones were identified from the NIH miniature pig BAC library, indicating that the library is an important source for identifying PERV-containing clones. A previous report indicated that a miniature pig genome might contain a minimum of 30 genomic regions containing PERVs [8], therefore, 150 PERV-containing clones should be found in the NIH miniature pig BAC library considering that the library has a 5X genome coverage. One of the reasons for this could be due to the complexity of PCR-based library screening techniques compared with the BAC filter-based methods, especially for screening the repetitive sequence as in PERVs. Along with this limitation, the genomic BAC library gave an important resource for identifying PERV-positive clones from NIH miniature pig. The full-length PERV nucleotide sequences identified in this study, together with the map information, can give important genomic information for the PERV researchers, especially for developing knockout strategy of the specific PERV. In conclusion, the PERV information in this study from the NIH miniature pig BAC library will provide valuable information for xenotransplantation studies, especially selection of pigs having specific PERV free animals. Also, this research can help the development of markers for screening the site-specific PERVs in the pig genome.

Acknowledgments

This work was supported by a Grant (no. 20070401034031) from BioGreen 21 program, Rural Development Administration, Republic of Korea and by a Grant (no. 20100023352) from the National Research Foundation of Korea, Republic of Korea. The authors gratefully acknowledge provision of pig radiation hybrid panel by Drs. Denis Milan and Martin Yerle (INRA, France).

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