SHOCK, Vol. 32, No. 1, pp. 80–88, 2009

CLONING AND CHARACTERIZATION OF ENDOGENOUS RETROVIRUSES ASSOCIATED WITH POSTINJURY STRESS SIGNALS IN LYMPHOID TISSUES

Deug-Nam Kwon, David G. Greenhalgh, and Kiho Cho

Department of Surgery, University of California, Davis and Shriners Hospitals for Children Northern California, Sacramento, California

Received 24 Mar 2008; first review completed 10 Apr 2008; accepted in final form 25 Aug 2008

ABSTRACT—Endogenous retroviruses (ERVs) constitute a significant fraction of the mouse and human genomes, ~10% and ~8%, respectively, and they are transmitted to offsprings in a Mendelian fashion. Recent reports implicated that certain ERVs participate in a range of disease processes. In this study, we examined injury-elicited changes in murine ERV (MuERV) expression in lymphoid tissues and characterized biological properties of the putative MuERVs isolates. Female C57BL/6J mice were subjected to ~18% total-body-surface-area burn injury. Four different lymphoid tissues (blood, bone marrow, spleen, and thymus) were collected at 24 h for reverse transcriptase–polymerase chain reaction analysis of MuERV expression by amplifying the 3′ U3 regions. Within each tissue examined, there was a unique pattern of injury-elicited changes in MuERV expression. From the 17 unique MuERV U3 clones isolated from all four tissues, nine were derived from injury-induced MuERVs, four from injury repressed, and four from no change. A survey of the C57BL/6J genome using all 17 U3 clones as probes produced 26 pertinent putative MuERVs, of which five were presumed to retain intact coding potentials for essential polypeptides. Biological properties (genomic location, tropism, transcriptional potential, coding potential, primer-binding site, recombination, and integration age) of each putative MuERV were characterized, and their relevance to injury response was discussed. The findings from this study suggest that injury-elicited stress signals either induce or repress specific MuERV populations in a lymphoid tissue–specific and probably cell type–specific manner. It warrants a further investigation into the roles of the injury-responsive MuERVs in postinjury pathogenic processes of the immune system.

KEYWORDS—Burn, murine endogenous retroviruses, spleen, thymus, bone marrow, blood cells, injury response

INTRODUCTION

Endogenous retroviruses (ERVs), which are footprints of germ line colonization of retroviruses, are present in all vertebrate genomes. Endogenous retroviruses constitute ~8% of human genome and ~10% of mouse genome, and they are passed down to the next generations in a Mendelian fashion (1, 2). A significant fraction of ERVs are known to be defective in regard to replication and/or viral gene expression because of mutations. However, some ERVs retain intact coding potentials for essential polypeptides and are replication competent (1). Transcription and translation of the proviral forms of ERVs are governed by the same biological processes as any other genetic coding units of the genome (2). In particular, ERVs' transcription is primarily controlled by the host's transcription machinery in association with a unique set of transcription regulatory elements within each ERV's U3 promoter. Alterations in ERV expression may be associated with a range of pathophysiological events, which are beneficial and/or harmful to the host, through viral replication, gene products, chromosomal rearrangement via recombination, and/or insertional mutagenesis.

The findings from recent studies provided evidence that ERVs may be involved in various disease processes (3–6). For instance, it has been reported that the proinflammatory properties of human ERV-W envelope protein, called syncytin, play a central role in demyelination of oligodendrocytes, leading to development of multiple sclerosis in humans (7). In addition, studies from our laboratory demonstrated that burn injury–elicited stress signals alter expression of murine ERVs (MuERVs) in distant organs (e.g., liver, lung) of mice (8–10). However, the precise pathophysiological roles of MuERVs in postinjury and other pathogenic processes are not clearly understood.

A complex network of signaling pathways are known to be activated in response to burn injury–elicited stress to the body (11, 12). Postinjury activation of a cascade of proinflammatory events in conjunction with initiation of an immunosuppressive state often predisposes patients to sepsis and multiple organ failure (13–16). The findings that there is an early reduction in the number of lymphocytes in various primary (e.g., bone marrow, thymus) and secondary (e.g., spleen, blood) lymphoid tissues of patients and experimental animals may explain, at least in part, the postinjury immunosuppressive state (17, 18). However, the detailed molecular and cellular mechanisms underlying the postinjury immune disorder have not yet been fully characterized.

In this study, we identified putative injury-responsive MuERV isolates in various lymphoid tissues, and their biological properties are determined. Further studies investigating the roles of MuERVs in postinjury immune disorder will shed a novel light into understanding causative agents/molecules/cells of injury-associated complications and development of novel therapeutic regimens.

Address reprint requests to Kiho Cho, PhD, DVM, Burn Research, Shriners Hospitals for Children and Department of Surgery, University of California, Davis, 2425 Stockton Blvd, Sacramento, CA 95817. E-mail: kcho@ucdavis.edu.

This study was supported by grants from Shriners of North America (no. 8680) and NIGMS (R01GM071360).

DOI: 10.1097/SHK.0b013e31818bc193

MATERIALS AND METHODS

Animal experiment

Female C57BL/6J mice from Jackson Laboratory (West Sacramento, Calif) were housed according to the guidelines of the National Institutes of Health. The Animal Use and Care Administrative Advisory Committee of the University of California, Davis, approved the experimental protocol. The burn protocol has been described previously (8). Briefly, under general anesthesia, an ~18% total-body-surface-area flame burn was generated on the shaved back of mice followed by immediate i.p. injections of 0.9% saline (1 mL) for resuscitation and buprenorphine (3 μg in 100 μL saline) for pain control. Control mice were shaved, anesthetized, and resuscitated, but not burned. Four mice from each group were killed by CO_2 inhalation for tissue (blood, bone marrow, spleen, and thymus) collection at 24 h after burn.

Reverse transcriptase–polymerase chain reaction

Total RNA isolation and cDNA synthesis were performed based on protocols described previously (8). Briefly, total RNA was extracted using an RNeasy kit (Qiagen, Valencia, Calif), and 100 ng of total RNA from each tissue sample was subjected to reverse transcription using Sensiscript reverse transcriptase (RT) (Qiagen). A set of primers, ERV-U1 (5′-CGG GCG ACT CAG TCT ATC GG-3′) and ERV-U2 (5′-CAG TAT CAC CAA CTC AAA TC-3′), were used to amplify the 3′ MuERV U3 region. These primers were previously used to amplify nonecotropic MuERV U3 regions (19).

Cloning and sequencing

Polymerase chain reaction (PCR)–amplified U3 fragments were purified using a Qiaquick Gel Extraction kit (Qiagen) and cloned into the pGEM-T Easy vector (Promega, Madison, Wis). Plasmid DNAs for sequencing analysis were prepared using a Qiaprep Spin Miniprep kit (Qiagen). Sequencing was performed at Molecular Cloning Laboratory (South San Francisco, Calif).

Multiple alignment and phylogenetic analyses

Initially, a total of 37 MuERV U3 clones were aligned using Vector NTI (Invitrogen, Carlsbad, Calif), and 17 unique U3 clones were identified (20). The neighbor-joining method within MEGA4 program was used for phylogenetic analysis (21, 22).

Tropism analysis (U3 promoter)

The putative tropism of the 17 unique U3 clones was determined by comparison to the reference sequences (direct repeat, insertion, and unique region) first reported by Tomonaga and Coffin (19, 23). A total of five direct repeats (1/1*, 3/3*, 4/4*, 5/5*, and 6/6*), a single 190bp insertion, and a unique sequence (2) served as references for the tropism analysis.

Profiling of transcription regulatory elements

Profiles of transcription regulatory elements within individual U3 promoters were determined using MatInspector program (Genomatix, Munich, Germany). The core similarity was set to 0.90, and the matrix similarity was optimized within the vertebrate matrix group (24).

In silico *cloning of putative MuERVs and open-reading-frame analyses*

Putative MuERV proviral sequences were identified by surveying the entire mouse (C57BL/6J) genome database from the National Center for Biotechnology Information (NCBI) using individual U3 promoter sequences as probes. Initially, the genomic U3 sequences with greater than 98% homology with respective U3 probes were marked for further mapping and cloning analyses. We then searched for putative MuERV sequences in the marked regions ranging from ~5 to ~9 kb and flanked by almost identical long terminal repeats (LTRs) at both the 5′ and 3′ ends. Subsequently, the open reading frames (ORFs) within each putative MuERV were analyzed using the ORF search feature within Vector NTI (Invitrogen). The parameter was set "ATG" as the start codon, and each candidate ORF was translated. The translation products were then compared with the murine leukemia virus references retrieved from NCBI (M17327, AY219567.2, and AF033811) using Vector NTI (Invitrogen). The criteria for defining the intactness of each proviral gene depended on the presence of p12 of *gag*, RT of *pol*, and SU (surface domain) of *env*. Individual genes were determined to be intact (+) if the aforementioned sequences were intact and the remaining amino acid sequence of each respective gene matched one of the reference sequences, while allowing for missense mutations. If the defining sequences were intact but the remaining gene sequences were defective, they were classified as partial (P). Defective (−) gene sequences contained deletions and/or premature stop codons in addition to alternative start codons leading to defective defining coding sequences.

Analyses of primer-binding site, recombination event, and integration age

A stretch of 18 bp, immediate downstream of the 3′-end of the 5′ U5 region, was examined to determine primer-binding sites (PBSs). The conserved PBS sequences for $tRNA^{Proline(P)}$ and $tRNA^{Glutamine(Q)}$ were used as references (25, 26). To analyze integration ages, 5′ and 3′ LTR sequences of each putative MuERV were compared using Vector NTI (Invitrogen). The integration age was calculated based on a formula of "0.13% mutation rate between two flanking LTRs per one million years (Myr)." In case there is only single nucleotide difference between two flanking LTRs, it is recorded as less than the estimated age in consideration of a potential error rate during cloning and sequencing. To examine the presence of genomic rearrangements between MuERVs, a stretch of four nucleotides flanking each MuERV was surveyed for a direct repeat, which is formed during the initial proviral integration. Any downstream recombination events result in two different sequences instead.

Tropism analysis (provirus)

Tropism traits of the putative full-length MuERVs with intact coding potentials were determined by *in silico* restriction fragment length polymorphism (RFLP) analysis using three restriction enzymes, *Bam*HI, *Eco*RI, and *Hind*III using Vector NT1 (Invitrogen). The RFLP data were compared with the reference profile for each tropism trait (ecotropic, xenotropic, polytropic and modified polytropic) (19, 23).

Quantitative analysis of RT-PCR data

Relative densities of individual RT-PCR fragments from each group were measured, normalized using the β-actin control, and presented as mean ± SD. Statistical significance was determined by Student *t*–test.

RESULTS

Injury-mediated differential alterations in MuERV expression in various lymphoid tissues

In this experiment, we examined whether burn-elicited stress signals change the expression of MuERVs in four different lymphoid tissues (blood, bone marrow, spleen, and thymus) at 24 h after burn by RT-PCR amplification of the 3′ U3 regions of the MuERV transcripts, which are relatively polymorphic compared with the rest (Fig. 1). In addition, the 3′ U3 is presumed to be identical to the 5′ U3, which serves as a promoter for ERV's transcription. There were two distinct amplified U3 fragments (~700 bp [labeled as "a": U3-a] and ~500 bp [labeled as "b": U3-b]) in the blood and bone marrow, and only one (a) fragment was present in the spleen. In addition to these two fragments (a and b), one additional fragment of ~450 bp (labeled as "c": U3-c) was amplified in the thymus (Fig. 1). It was evident that the U3-b fragment was induced ($P < 0.05$) in all four tissues at 24 h after injury, whereas the U3-a fragment was significantly repressed ($P < 0.05$) in the blood and somewhat lesser degree in the thymus. There was no change in U3-a fragment in the bone marrow. In addition, the U3-c fragment was substantially induced ($P < 0.05$) in the thymus after injury. It is interesting to note that the patterns of baseline expression as well as of injury-mediated alterations in MuERV expression are unique for individual lymphoid tissues examined. Because the gut-associated immune system has been implicated in the postburn pathogenesis, we examined whether burn-elicited stress signals altered the expression profile of MuERVs in the mesenteric lymph node at 24 h after burn. Semiquantitative RT-PCR analysis revealed no significant changes in the postburn expression pattern of MuERVs in the mesenteric lymph node (data not shown). Previous studies have demonstrated that a substantial degree of postburn immune dysregulation is associated with pathological changes in the spleen at 7 to 10 days after burn, such as increased proliferative activity in the red

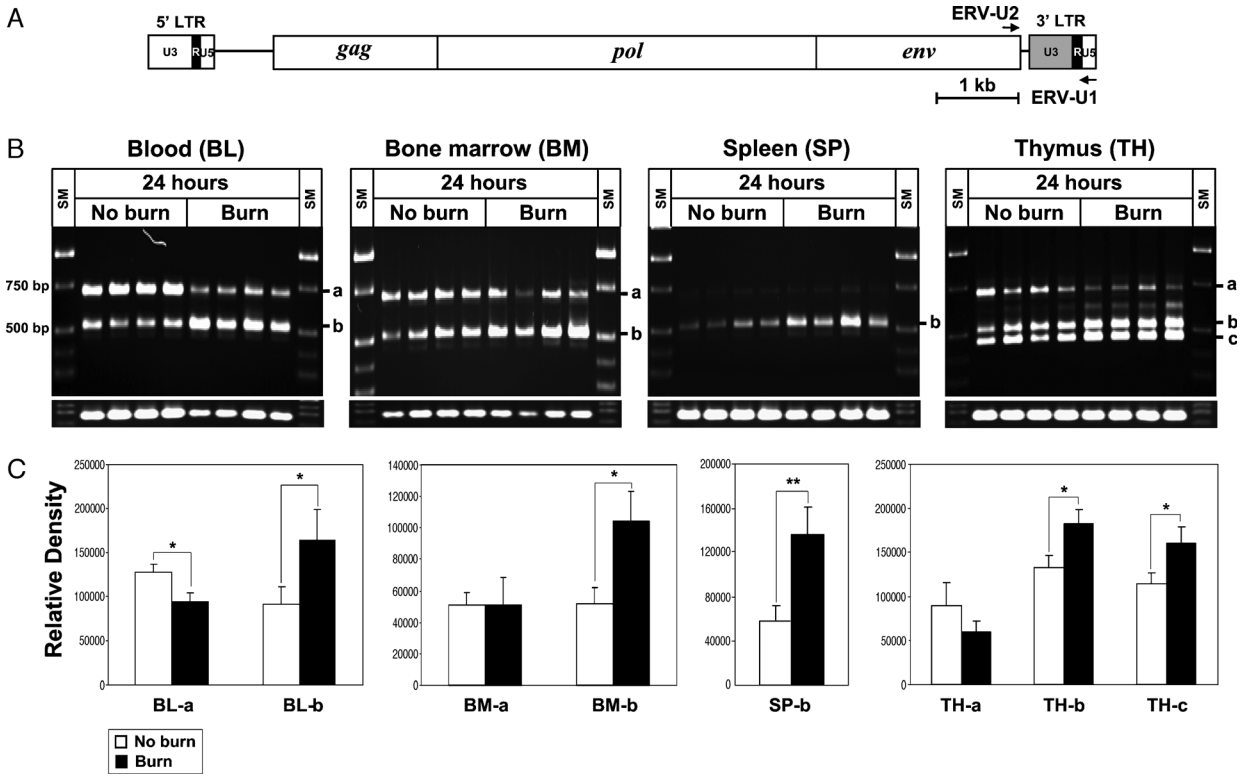


FIG. 1. **Burn-mediated differential changes in MuERV expression in lymphoid tissues**. A, Schematic representation of primer locations on a typical retroviral provirus. A set of primers (ERV-U2 and ERV-U1) flanking the 3′ U3 region is indicated by arrows. B, RT-PCR analysis of MuERV expression in lymphoid tissues after burn. Postburn changes in MuERV expression were analyzed by RT-PCR by amplifying the 3′ U3 region in the blood, bone marrow, spleen, and thymus. β-Actin serves as an internal control. C, Quantitative analysis of RT-PCR data. Relative densities of fragments from each group were measured, normalized using the β-actin control, and presented as mean ± SD; * and ** indicate statistical significance (*$P < 0.05$; **$P < 0.001$). a, b, and c represent different sizes of amplified U3 fragments.

pulp. In this experiment, we investigated whether postburn changes in the MuERV expression profile in the spleen parallel the pathological changes at 7 days. No significant changes in the expression profile of MuERVs were observed at 7 days after burn in contrast to a marked change at 1 day (data not shown). The findings from this study suggest that burn-elicited stress signals differentially modulate the expression of specific groups of MuERVs, depending on lymphoid tissue type and time after injury. We then investigated further to determine the biological properties of the burn-associated putative MuERVs.

Sequence analysis of U3 clones isolated from burn-induced and burn-repressed MuERVs

To investigate genetic variations among three distinct groups of amplified U3 fragments (injury-induced [I], injury-repressed [II], and no change [III]), cloning and sequence analyses were performed (Fig. 2A). At least two U3 clones isolated from each experimental group (e.g., blood/burn/U3-a fragment) were included for a total of 37 U3 clones subjected to an initial alignment analysis, which resulted in 17 unique U3 clones with seven different sizes ranging from 346 to 615 bp. Analysis of the burn-induced U3-b fragment from all four tissues yielded two U3 sizes (392 and 406 bp), and the U3-c fragment from the thymus was represented by two sizes (346 and 361 bp) of U3 clones (Fig. 2A). The U3 clones isolated from the U3-b fragment were predominantly 406 bp in size (16 of initial 20 U3 clones analyzed). In addition, three (600, 601, and 615 bp) different sizes of U3 clones were identified from the U3-a fragments that were repressed in the blood and thymus, and no change in the bone marrow. It is interesting to note that all four U3 clones isolated from the bone marrow U3-a fragment were different from the ones derived from the U3-a fragments of the blood and thymus. Subsequently, the 17 unique U3 clones identified in this study were subjected to multiple alignment followed by phylogenetic analysis. Sequences of both the 5′ and 3′ ends were well conserved among the U3 clones aligned, in contrast to the middle region, which was highly polymorphic, including a 190bp insertion in some clones. The branching patterns within the phylogenetic tree established from the alignment data were unique for the U3 clones derived from the burn-induced (I) MuERV fragments compared with the ones derived from burn-repressed (II) and no-change (III) fragments, as predicted primarily based on their sizes (Fig. 2B). It is likely that the difference in sizes of U3 clones (U3-a fragment > U3-b/c fragments) was one of the key determinants of these branching patterns. The results from this study revealed that a diverse group of putative MuERVs, which harbor the unique burn-associated U3 sequences, may participate in a network of events responsible for the postburn pathogenesis.

Tropism traits of MuERV U3 clones

It has been documented that MuERV tropism is closely linked to the sequence characteristics of the U3 promoter region. The tropism traits of the 17 unique U3 clones

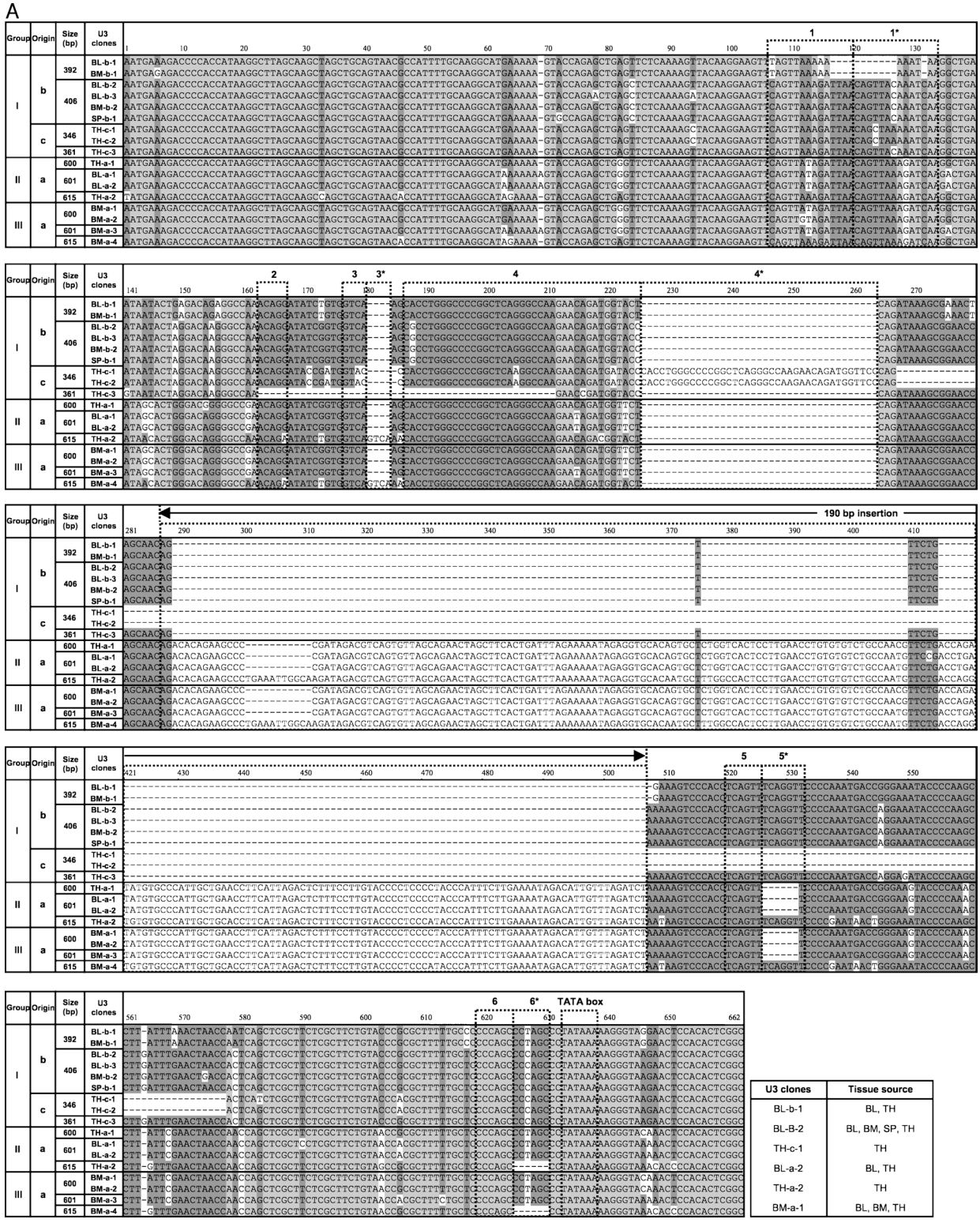


FIG. 2. **Multiple alignment and phylogenetic analyses of U3 clones isolated from burn-induced and burn-repressed/no-change MuERVs**. A, Multiple alignment analysis of U3 clones isolated from burn-induced and burn-repressed/no-change MuERV U3 fragments. The 17 unique MuERV U3 clones isolated from blood, bone marrow, spleen, and thymus of burn and/or no-burn mice were subjected to multiple alignment analysis. Different gray scales and a dash indicate various levels of sequence homology (gray, 100% homology; dark gray, partial/conserved; white, no homology; and dash, absence of sequence). Distinct sequence features are indicated (dotted box: direct repeat, unique region, 190bp insertion, and TATA box). The table on the bottom right lists all tissue types that share the unique U3 clones. I (U3 clones derived from burn-induced MuERVs), II (U3 clones derived from burn-repressed MuERVs), and III (U3 clones derived from no-change MuERVs). B, Phylogenetic analyses of U3 clones isolated from burn-induced and burn-repressed/no-change MuERV U3 fragments. The phylogenetic tree was established using the neighbor-joining method. Branch lengths are proportional to the distance between the taxa, which are drawn to scale. The values at the branch nodes indicate the percentage support for a particular branching.

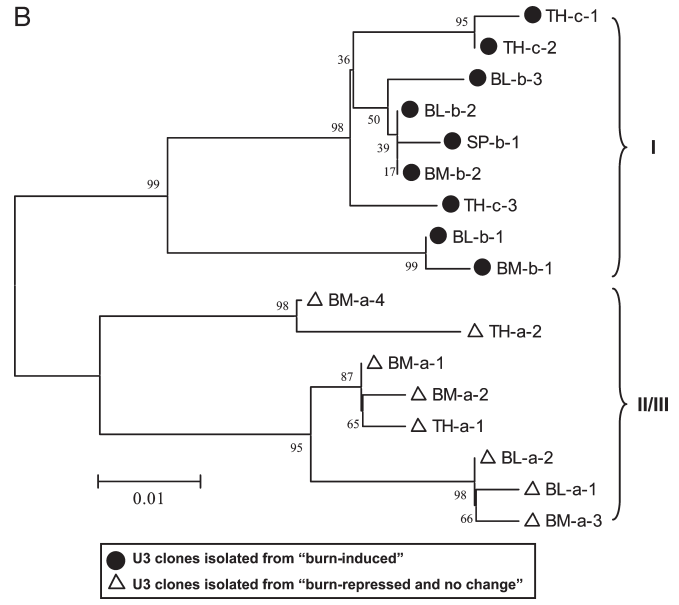


FIG. 2. ***(continued)***

identified in this study were determined by comparison analysis using the reference features (direct repeat, unique region, and 190bp insertion) and the accompanying protocol first reported by Tomonaga and Coffin (19, 23) (Table 1). A total of five direct repeats (1/1*, 3/3*, 4/4*, 5/5*, and 6/6*), one 190bp insertion, and one unique sequence (2) were identified among the U3 clones examined (Fig. 2A). The sequence variations revealed by alignment analysis of the 17 U3 clones were somewhat concurrent with presence and/or absence of these reference features. For instance, both the 3/3* direct repeat and 190bp insertion were present only in the BM-a-4 and TH-a-2 U3 clones, whereas the 4/4* direct repeat was identified only in the TH-c-1 and TH-c-2 U3 clones. Table 1 summarizes the tropism trait of each U3 clone; there were six polytropic and nine xenotropic, and there were insufficient data to determine tropism traits of two U3 clones (TH-a-2 and BM-a-4). Interestingly, correlation between tropism traits and burn responsiveness (induced or repressed) of individual U3 clones examined was observed; U3 clones isolated from burn-induced U3-b/c fragments were xenotropic, whereas ones from burn-repressed/no response U3-a fragments were predominantly polytropic (6 of 8). The tropism trait data acquired from this study need to be confirmed by an *in vitro* infection assay; however, these findings suggest that the burn-associated putative MuERVs may be able to infect mouse cells and/or other cell types derived from nonmouse species.

Transcriptional potentials of MuERV U3 clones/promoters

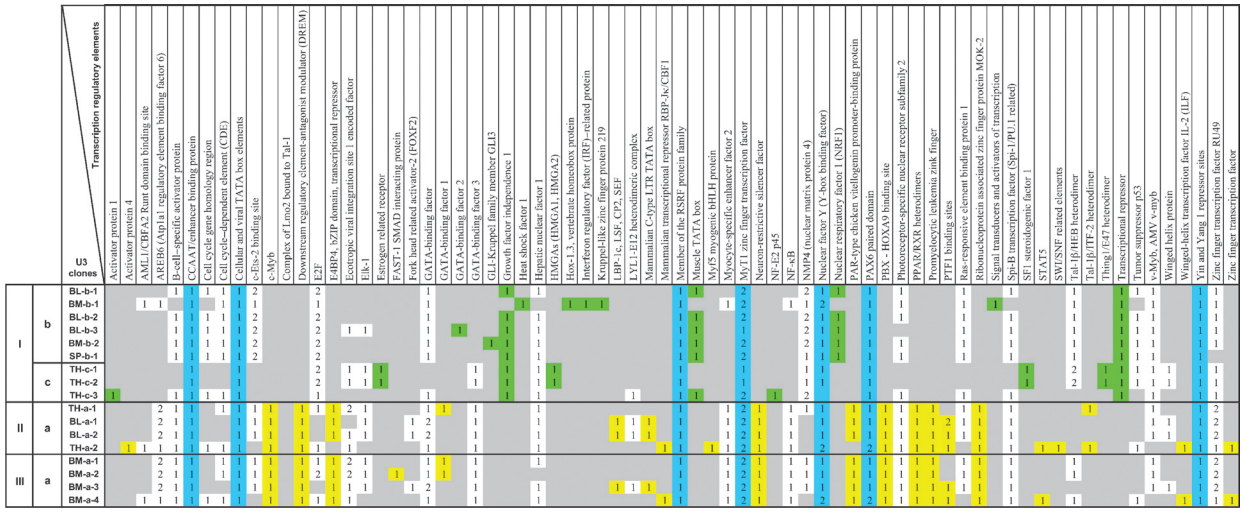
To examine the transcription potentials of the 17 unique MuERV U3 clones/promoters, the profile of putative transcription regulatory elements within each U3 clone was determined (Table 2). Among a total of 72 putative elements identified among the U3 clones analyzed, seven elements (blue), including CCAAT/enhancer, TATA box, and PAX6 paired domain, were shared by all U3 clones examined. On the other hand, 17 elements (green), such as transcriptional repressor and estrogen-related receptor, were present only in the U3 clones derived from burn-induced fragments, and 22 elements (yellow), including PPAR/RXR heterodimer and c-Myb, were mapped exclusively on the U3 clones derived

TABLE 1. **Summary of tropism traits of 17 unique MuERV U3 clones**

Group	U3 clones	Unique sequence/Direct repeat*					Tropism
		1/1*	2	4/4*	5/5*	6/6*	
	BL-b-1	—	*X-I,* II, IV, Poly	*X-I*	X-II, III, IV	—	**X-I**
	BM-b-1	—	*X-I,* II, IV, Poly	*X-I*	X-II, III, IV	—	**X-I**
	BL-b-2	X-III	X-I, *II,* IV, Poly	*X-II,* IV	*X-II,* III, IV	X-I, *II,* III, IV, Poly	**X-II**
	BL-b-3	X-III	X-I, *II,* IV, Poly	*X-II,* IV	*X-II,* III, IV	X-I, *II,* III, IV, Poly	**X-II**
I	**BM-b-2**	X-III	X-I, *II,* IV, Poly	*X-II,* IV	*X-II,* III, IV	X-I, *II,* III, IV, Poly	**X-II**
	SP-b-1	X-III	X-I, *II,* IV, Poly	*X-II,* IV	*X-II,* III, IV	*X-II,* III, IV, Poly	**X-II**
	TH-c-1	—	X-I, *II,* IV, Poly	*X-II,* IV	—	X-I, *II,* III, IV, Poly	**X-II**
	TH-c-2	—	X-I, *II,* IV, Poly	*X-II,* IV	—	X-I, *II,* III, IV, Poly	**X-II**
	TH-c-3	*X-III*	*X-III,* Poly	*X-III*	X-II, *III,* IV, Poly	X-I, II, *III,* IV, Poly	**X-III**
	TH-a-1	*Poly*	X-I, II, IV,*Poly*	*Poly*	—	—	**P-II**
II	**BL-a-1**	*Poly*	X-I, II, IV,*Poly*	*Poly*	—	—	**P-II**
	BL-a-2	*Poly*	X-I, II, IV,*Poly*	*Poly*	—	—	**P-II**
	TH-a-2	Poly	—	X-I, Poly	X-II, III, IV, Poly	X-I, II, III, Poly	**?**
III	**BM-a-1**	*Poly*	X-I, II, IV,*Poly*	*Poly*	—	—	**P-II**
	BM-a-2	*Poly*	X-I, II, IV,*Poly*	*Poly*	—	—	**P-II**
	BM-a-3	*Poly*	X-I, II, IV,*Poly*	*Poly*	—	—	**P-II**
	BM-a-4	Poly	—	X-I, Poly	X-II, III, IV, Poly	X-I, II, III, Poly	**?**

Em dash indicates absence of homology with reference sequences. Underlined italicized data represent the reference type closest to each U3 clone examined. I (U3 clones derived from burn-induced MuERVs), II (U3 clones derived from burn-repressed MuERVs), and III (U3 clones derived from no-change MuERVs).
*indicates direct repeat; X, xenotropic; Poly, polytropic.

TABLE 2. **Profiles of transcription regulatory elements in 17 unique MuERV U3 promoters/clones**



Numbers in the box indicate frequency of each element. Gray shade indicates no occurrence of specific elements. Different colors indicate elements shared by all U3 clones (blue), mapped only in U3 clones derived from burn-induced (green) MuERVs, or only in U3 clones from burn-repressed/no-change MuERVs (yellow). I (U3 clones derived from burn-induced MuERVs), II (U3 clones derived from burn-repressed MuERVs), and III (U3 clones derived from no-change MuERVs).

from burn-repressed and/or no-change U3 fragments. In addition, certain elements were identified only in a specific U3 clone, for instance, a binding element for signal transducers and activators of transcription within the BM-b-1 U3 clone derived from burn-induced U3 fragment of bone marrow. The finding of unique profiles of transcription regulatory elements within U3 clones derived from burn-induced MuERVs compared with ones from burn-repressed/no-change MuERVs suggests that they will respond differentially to an altered transcriptional environment associated with injury-elicited stress signals. In addition, the unique transcriptional environment of the individual lymphoid tissues in conjunction with the profile of transcription regulatory elements within the U3 promoter of each putative MuERV might be directly linked to the differential baseline expression as well as postburn modulation of MuERVs in these tissues.

Cloning of putative MuERVs using U3 clones as probes and characterization of their biological properties

To identify the putative MuERVs harboring each U3 clone, the NCBI C57BL/6J genome database was surveyed using the U3 clones as probes. The putative MuERVs with a greater than 98% homology with respective probes were mapped on the genome (chromosomal location and orientation) and cloned *in silico*. A total of 26 putative MuERVs, ranging from 5,312 to 9,054 bp in proviral size, were cloned, and they were distributed throughout the genome except for chromosomes 9, 14, 15, 16, 17, 18, 19, X, and Y (Table 3). For each putative MuERV isolate, a complete proviral sequence plus flanking host sequences (50 bp from both sides) was subjected to the following analyses: PBS, ORF (coding potential), direct repeat (recombination), tropism trait, and LTR mutation rate (integration age).

To determine coding potentials of each putative MuERV isolate, the ORFs for *gag*, *pol*, and *env* polypeptides were surveyed using murine leukemia virus references with intact coding potentials. Interestingly, five of the 26 putative MuERVs were full-length retaining coding potentials for all three essential polypeptides (*gag*, *pol*, and *env*), whereas the rest had defective and/or partial ORFs for these polypeptides. Some putative MuERVs capable of encoding *gag* and/or *env* proteins (e.g., BL-a-2.1, TH-c-3.2) may exert their biological roles via these gene products. Alternatively, in the presence of full-length helper viruses, the defective/incomplete MuERVs may also be able to replicate.

A stretch of 18bp sequence immediately downstream of the 5′ U5 region was examined to determine the PBS for each putative MuERV isolate using reference sequences, and it turned out that all had the same PBS, $tRNA^{Glutamine(Q)}$ (indicated as "Q" in Table 3) (26). The $tRNA^{Glutamine(Q)}$ PBS is reported to be used by the RT of polytropic and modified polytropic MuERVs during replication.

To determine whether there were genetic rearrangements in the putative MuERV isolates since their initial integrations into the genome, the direct repeat sequences flanking the 5′ and 3′ ends of each proviral sequence were surveyed. It has been documented that direct repeats are formed during the initial integration event of any retroviruses, and any genomic rearrangements involving proviral sequences, primarily recombination via LTRs, replace the direct repeats with two different sequences (27). Seven of 26 putative MuERVs did not retain direct repeats at their integration sites, suggesting they are recombinant MuERVs, whereas the rest had a direct repeat of 4 bp. Interestingly, two of the presumed to be recombinant MuERVs retained all three ORFs intact.

The integration ages of these putative MuERVs were calculated based on the mutation rate between two flanking LTRs. Integration ages of five putative MuERVs were determined, ranging from 1.0175 Myr (0.1323% mutation rate) to 3.0893 Myr (0.4016% mutation rate). Because 14 of

TABLE 3. **Biological properties of 26 putative MuERVs**

MuERV	Ch	Location (orientation)	Size (bp)	PBS	ORF			Direct repeat	Integration age (Myr) [mutation rate]
					gag	*pol*	*env*		
BL-a-2.1	1	133470113-133479166 (+)	9054	Q	–	+	+	ACAC	3.0893 [0.4016]
TH-a-1.2	2	C15947290-15938249 (−)	9042	Q	–	+	+	ATTG	<1 [0]
TH-c-3.2	2	C156055046-156047997 (−)	7050	Q	+	–	+	CCAG	<1 [0]
TH-a-1.3a	3	67184007-67191723 (+)	7717	Q	+	–	–	ACTT	<1 [0]
TH-a-1.3b	3	152260526-152269568 (+)	9043	Q	+	+	+	ATGT	<1 [0]
BL-a-2.4a	4	C15169957-15163352 (−)	6606	Q	–	–	–	CAGG	1.0381 [0.1350]
BL-a-2.4b	4	133431467-133436778 (+)	5312	Q	+	–	–	AACA	<1 [0]
TH-c-1.4	4	132368033-132373699 (+)	5667	Q	–	–	–	CCTT	<1 [0]
TH-a-1.5a	5	24740764-24749735 (+)	8972	Q	+	P	P	ATAC	<1 [0]
TH-a-1.5b	5	43496191-43505229 (+)	9039	Q	+	+	+	ATAT/TTAT	ND
TH-a-1.5c	5	C110148316-110140851 (−)	7466	Q	P	P	–	ATAG	<1 [0]
TH-a-1.5d	5	122453464-122460825 (+)	7362	Q	–	P	–	GATG	<1 [0]
BL-a-2.6	6	C73223659-73216301 (−)	7359	Q	+	–	–	ACAA/ACAC	ND
BL-a-2.7	7	64005512-64014552 (+)	9041	Q	+	+	+	CCTG	<1 [0]
TH-c-1.8	8	93776396-93782063 (+)	5668	Q	–	–	–	ATAT	1.5795 [0.2053]
BL-a-2.8	8	126050806-126058167 (+)	7362	Q	+	–	–	GGAA/GGTG	ND
BM-a-2.10a	10	22423694-22432738 (+)	9045	Q	+	+	–	CTGC/TTGC	ND
BM-a-2.10b	10	C4628826-4619788 (−)	9039	Q	P	–	–	ACAG/TTAG	ND
BM-a-2.11a	11	C60402203-60394844 (−)	7360	Q	–	P	–	ACAC	<1 [0]
BM-a-2.11b	11	76365003-76374047 (+)	9045	Q	+	+	+	AGGG/TTGG	ND
BM-a-2.11c	11	102899753-102908794 (+)	9042	Q	P	P	+	AAAC/GAAA	ND
BM-a-2.11d	11	86698511-86707551 (+)	9041	Q	+	+	+	ACAC	1.0381 [0.1350]
BM-a-2.11e	11	5905655-5911518 (+)	5864	Q	–	–	–	CAGT	1.0175 [0.1323]
TH-a-1.12	12	70465411-70474308 (+)	8898	Q	P	+	+	AGAC	<1 [0]
BL-b-2.13a	13	C68392677-68383991 (−)	8687	Q	P	+	+	GTAC	<1 [0]
TH-a-1.13b	13	21819875-21827661(+)	7787	Q	+	+	P	CTAC	<1 [0]

Gray shade indicates full-length MuERVs with intact coding potentials; +, intact; −, defective; P, partial; Ch, chromosome; Q, $tRNA^{Glutamine}$.

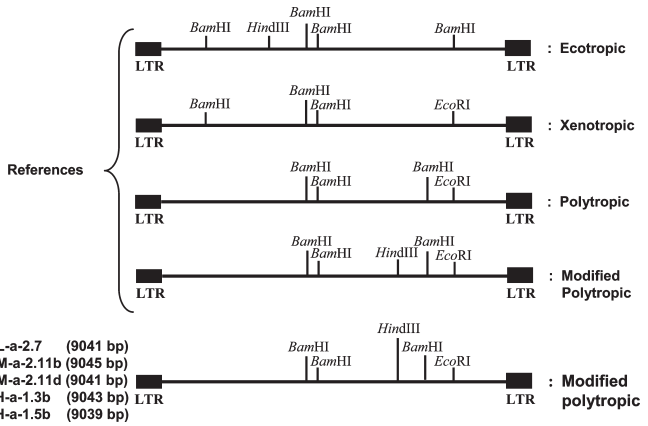


FIG. 3. **Tropism analysis of putative MuERV isolates with intact coding potential for essential polypeptides**. Tropism traits of the five putative full-length MuERVs were determined by *in silico* RFLP analysis using *Bam*HI, *Eco*RI, and *Hin*dIII restriction enzymes. Relative locations of individual restriction enzyme sites were mapped on one of five putative MuERV isolates, and all of them were presumed to be modified polytropic. Reference RFLP patterns of ecotropic, xenotropic, polytropic, and modified polytropic MuERVs are presented.

them had no mismatch between flanking LTRs, their integration ages were arbitrarily recorded as less than 1 Myr. On the other hand, the integration ages of putative recombinant isolates were not determined because the genetic rearrangement events skew the LTR mutation rate.

The tropism traits of the five putative MuERVs with intact coding potentials for essential polypeptides were determined by RFLP analysis using three enzymes (*Bam*HI, *Eco*RI, and *Hin*dIII). Comparison analysis using the reference RFLP profiles (ecotropic, xenotropic, and polytropic) revealed that all five putative MuERVs were presumed to be modified polytropic (Fig. 3). The results obtained from this study provide baseline information for understanding the biological properties of burn-associated putative MuERVs. In particular, it suggests that some of these putative MuERVs are replication competent and are able to infect mouse cells and/or cells of other species.

DISCUSSION

Burn injury–elicited stress signals are associated with pathogenic phenotypes of depletion and/or clonal expansion of certain subsets of the immune cell pool, leading to proinflammatory

as well as anti-inflammatory immune responses (10, 28). There is a limited understanding of mechanisms controlling postburn immune disorder, such as death of immune cells followed by compensatory proliferation (29, 30). In an attempt to investigate whether MuERVs participate in the events underlying the postburn phenotypic changes in lymphoid tissues, we examined alterations in MuERV expression in various lymphoid tissues (blood, bone marrow, spleen, and thymus). Biological properties of the putative MuERVs isolated using the U3 clones as probes, which were derived from the burn-induced or burn-repressed/no-change MuERVs, were characterized. There were five key findings from this study. First, the profile of postburn changes in MuERV expression was unique for each tissue examined. Second, postburn induction was evident for some MuERVs, whereas another group of MuERVs was repressed. Third, in addition to the difference in sizes, the U3 clones from the burn-induced MuERVs had unique genomic features, tropism traits, and transcriptional potentials compared with the U3 clones from the burn-repressed/no-change MuERVs. Fourth, five of the 26 putative MuERV isolates retained intact coding potentials for essential polypeptides for viral assembly and replication, and the rest had defective and/or partial ORFs. Fifth, two putative MuERVs, presumed to be recombinants, retained intact ORFs for all three essential polypeptides.

It was interesting to observe that there were distinct profiles of postinjury MuERV responses in the four different lymphoid tissues examined. In addition, there were unique profiles of transcription regulatory elements in the U3 clones derived from burn-induced MuERVs compared with ones from burn-repressed/no-change MuERVs. These findings suggest that the baseline as well as postinjury transcriptional environment within each lymphoid tissue in conjunction with the profile of the resident immune cell population may be key contributing factors for the unique MuERV response. Alternatively, due to different physical locations and/or immunologic roles of these lymphoid tissues in the body, each tissue may be subjected to a unique set of burn-elicited stress signals, leading to differential MuERV responses. Further investigation into the postinjury MuERV response in individual immune cell types (e.g., B lymphocytes, T lymphocytes, macrophages) from different tissues may be needed to better understand the roles of MuERVs in postinjury immune disorder, such as thymic apoptosis and unregulated clonal expansion of lymphocyte subsets (31, 32).

There are four potential mechanisms of how alterations in MuERV expression contribute to burn-mediated immune disorder. First, the primary roles of certain MuERVs (induced and repressed) in immune regulation may reside in their ability to encode retroviral proteins, such as gp70 envelope, p30 capsid, and p12, and these proteins may participate in specific signaling events controlling the immune system. Second, five full-length MuERV isolates identified in this study have coding potentials for all essential proteins for the assembly of potentially pathogenic virus particles and subsequent infection. Third, the defective MuERV isolates, some of which are similar to mouse AIDS virus, may become pathogenic in the presence of a helper virus (33–36). Fourth, changes in transcriptional activities of certain MuERV loci on the genome may affect the expression of neighboring genes responsible for the immune function via enhancers and/or negative regulatory elements.

The results obtained from this study provide evidence that stress signals elicited from burn can alter the expression of a specific set of MuERVs, both full-length and defective/subgenomic, in a lymphoid tissue type–specific manner. It is possible that the postburn MuERV expression profile in the lymphoid tissues might be variable, depending on cell types and location of tissues, such as a unique postburn MuERV expression profile in B lymphocytes from mesenteric lymph nodes. The differential MuERV responses to burn-elicited stress signals depending on lymphoid tissue and/or cell type might be directly associated with their roles in a network of signaling events leading to phenotypic changes in the immune system as well as distant organs. The outcomes from this study warrant further investigation into the specific mechanisms of how these MuERVs contribute to postburn immune disorder, such as induction of inflammatory mediators, as well as distant organ failure.

Understanding the effects and underlying mechanisms of postburn modulation of MuERVs will broaden insights into the complex network of the burn-associated disease processes. It may ultimately lead to the development of a novel therapeutic protocol, such as antiretroviral treatment using anti–human ERV siRNAs and/or antiretroviral agents currently prescribed for the control of HIV, in combination with current regimens for burn patients.

REFERENCES

1. Clausen J: Endogenous retroviruses and MS: using ERVs as disease markers. *Int MS J* 10:22–28, 2003.
2. Larsson E, Andersson G: Beneficial role of human endogenous retroviruses: facts and hypotheses. *Scand J Immunol* 48:329–338, 1998.
3. Urnovitz HB, Murphy WH: Human endogenous retroviruses: nature, occurrence, and clinical implications in human disease. *Clin Microbiol Rev* 9:72–99, 1996.
4. Karlsson H, Bachmann S, Schroder J, McArthur J, Torrey EF, Yolken RH: Retroviral RNA identified in the cerebrospinal fluids and brains of individuals with schizophrenia. *Proc Natl Acad Sci U S A* 98:4634–4639, 2001.
5. Conrad B, Weissmahr RN, Boni J, Arcari R, Schupbach J, Mach B: A human endogenous retroviral superantigen as candidate autoimmune gene in type I diabetes. *Cell* 90:303–313, 1997.
6. Talal N, Garry RF, Schur PH, Alexander S, Dauphinee MJ, Livas IH, Ballester A, Takei M, Dang H: A conserved idiotype and antibodies to retroviral proteins in systemic lupus erythematosus. *J Clin Invest* 85:1866–1871, 1990.
7. Antony JM, van Marle G, Opii W, Butterfield DA, Mallet F, Yong VW, Wallace JL, Deacon RM, Warren K, Power C: Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination. *Nat Neurosci* 7:1088–1095, 2004.
8. Cho K, Adamson LK, Greenhalgh DG: Induction of murine AIDS virus-related sequences after burn injury. *J Surg Res* 104:53–62, 2002.
9. Cho K, Greenhalgh DG: Injury-associated induction of two novel and replication-defective murine retroviral RNAs in the liver of mice. *Virus Res* 93:189–198, 2003.
10. Cho K, Pham TN, Greenhalgh DG: CD14-dependent modulation of transcriptional activities of endogenous retroviruses in the lung after injury. *Virus Genes* 30:5–12, 2005.
11. Ipaktchi K, Mattar A, Niederbichler AD, Hoesel LM, Vollmannshauser S, Hemmila MR, Su GL, Remick DG, Wang SC, Arbabi S: Attenuating burn wound inflammatory signaling reduces systemic inflammation and acute lung injury. *J Immunol* 177:8065–8071, 2006.
12. Klein D, Einspanier R, Bolder U, Jeschke MG: Differences in the hepatic signal transcription pathway and cytokine expression between thermal injury and sepsis. *Shock* 20:536–543, 2003.
13. Teodorczyk-Injeyan JA, Sparkes BG, Mills GB, Peters WJ, Falk RE:

Impairment of T cell activation in burn patients: a possible mechanism of thermal injury-induced immunosuppression. *Clin Exp Immunol* 65:570–581, 1986.
14. Saffle JR, Sullivan JJ, Tuohig GM, Larson CM: Multiple organ failure in patients with thermal injury. *Crit Care Med* 21:1673–1683, 1993.
15. Baue AE, Durham R, Faist E: Systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), multiple organ failure (MOF): are we winning the battle? *Shock* 10:79–89, 1998.
16. Still JM Jr, Belcher K, Law EJ: Experience with polymicrobial sepsis in a regional burn unit. *Burns* 19:434–436, 1993.
17. Markley K, Smallman ET: Effect of thermal trauma on numbers and function of T and B cells from mouse spleen. *Int Arch Allergy Appl Immunol* 54:238–246, 1977.
18. Organ BC, Antonacci AC, Chiao J, Chiao J, Kumar A, de Riesthal HF, Yuan L, Black D, Calvano SE: Changes in lymphocyte number and phenotype in seven lymphoid compartments after thermal injury. *Ann Surg* 210:78–89, 1989.
19. Tomonaga K, Coffin JM: Structures of endogenous nonecotropic murine leukemia virus (MLV) long terminal repeats in wild mice: implication for evolution of MLVs. *J Virol* 73:4327–4340, 1999.
20. Xiong Y, Eickbush TH: Origin and evolution of retroelements based upon their reverse transcriptase sequences. *EMBO J* 9:3353–3362, 1990.
21. Kumar S, Tamura K, Nei M: MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5:150–163, 2004.
22. Saitou N, Nei M: The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425, 1987.
23. Tomonaga K, Coffin JM: Structure and distribution of endogenous nonecotropic murine leukemia viruses in wild mice. *J Virol* 72:8289–8300, 1998.
24. Quandt K, Frech K, Karas H, Wingender E, Werner T: MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 23:4878–4884, 1995.
25. Harada F, Peters GG, Dahlberg JE: The primer tRNA for Moloney murine leukemia virus DNA synthesis. Nucleotide sequence and aminoacylation of tRNAPro. *J Biol Chem* 254:10979–10985, 1979.
26. Nikbakht KN, Ou CY, Boone LR, Glover PL, Yang WK: Nucleotide sequence analysis of endogenous murine leukemia virus-related proviral clones reveals primer-binding sites for glutamine tRNA. *J Virol* 54:889–893, 1985.
27. Sverdlov ED: Retroviruses and primate evolution. *Bioessays* 22:161–171, 2000.
28. Schwacha MG, Holland LT, Chaudry IH, Messina JL: Genetic variability in the immune-inflammatory response after major burn injury. *Shock* 23:123–128, 2005.
29. Schwacha MG, Schneider CP, Chaudry IH: Differential expression and tissue compartmentalization of the inflammatory response following thermal injury. *Cytokine* 17:266–274, 2002.
30. Sjoberg T, Mzezewa S, Jonsson K, Salemark L: Immune response in burn patients in relation to HIV infection and sepsis. *Burns* 30:670–674, 2004.
31. Ginaldi L, De Martinis M, Monti D, Franceschi C: The immune system in the elderly: activation-induced and damage-induced apoptosis. *Immunol Res* 30:81–94, 2004.
32. Fitch FW, McKisic MD, Lancki DW, Gajewski TF: Differential regulation of murine T lymphocyte subsets. *Annu Rev Immunol* 11:29–48, 1993.
33. Aziz DC, Hanna Z, Jolicoeur P: Severe immunodeficiency disease induced by a defective murine leukaemia virus. *Nature* 338:505–508, 1989.
34. Chattopadhyay SK, Morse HC 3rd, Makino M, Ruscetti SK, Hartley JW: Defective virus is associated with induction of murine retrovirus-induced immunodeficiency syndrome. *Proc Natl Acad Sci U S A* 86:3862–3866, 1989.
35. Kubo Y, Nakagawa Y, Kakimi K, Matsui H, Higo K, Wang L, Kobayashi H, Hirama T, Ishimoto A: Molecular cloning and characterization of a murine AIDS virus-related endogenous transcript expressed in C57BL/6 mice. *J Gen Virol* 75(pt 4):881–888, 1994.
36. Ter-Grigorov VS, Krifuks O, Liubashevsky E, Nyska A, Trainin Z, Toder V: A new transmissible AIDS-like disease in mice induced by alloimmune stimuli. *Nat Med* 3:37–41, 1997.