Published in final edited form as:

J Autoimmun. 2012 June ; 38(4): 361–368. doi:10.1016/j.jaut.2012.03.002.

***Sgp3* and TLR7 Stimulation Differentially Alter the Expression Profile of Modified Polytropic Retroviruses Implicated in Murine Systemic Lupus**

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Abstract

The envelope glycoprotein, gp70, of endogenous retroviruses represents one of the major nephritogenic autoantigens implicated in murine systemic lupus erythematosus. Among different endogenous retroviruses (ecotropic, xenotropic and polytropic), lupus-prone mice express remarkably high levels of modified polytropic (mPT) retroviruses, which are controlled by the *Sgp3* (*serum gp70 production*) locus. To define the contribution of the *Sgp3* locus derived from lupus-prone mice to the expression of the specific mPT proviruses, the genetic origin of different mPT viruses expressed in livers and thymi of wild-type and *Sgp3* congenic C57BL/6 mice was determined through clonal analysis of their transcripts. Among 13 mPT proviruses present in the C57BL/6 genome, only 3 proviruses (*Mpmv6*, *Mpmv10* and *Mpmv13*) were selectively but differentially expressed in livers and thymi. This was likely a result of co-regulated expression with host genes because of their integration in the same transcriptional direction. In contrast, *Sgp3* induced the steady-state expression of an additional select group of mPT proviruses and, after stimulation of TLR7, the highly upregulated expression of a potentially replication-competent mPT virus *Mpmv4*. These results indicated that the expression of distinct subpopulations of mPT retroviruses was regulated by *Sgp3*- and TLR7-dependent mechanisms. The induction of potentially replication-competent mPT viruses and the upregulation of one such virus after stimulation with TLR7 in *Sgp3* congenic mice further highlight the implication of *Sgp3* in autoimmune responses against nephritogenic serum gp70 through the activation of TLR7.

Keywords

Systemic lupus erythematosus; Endogenous retrovirus; Toll-like receptor

1. Introduction

Endogenous retroviruses are classified as ecotropic, xenotropic or polytropic according to the host range defined by their respective surface gp70 proteins. Furthermore, based on

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differences in their gp70 nucleotide sequences [1], the polytropic proviruses have been divided into two subgroups, termed polytropic (PT) and modified PT (mPT). The retroviral *env* (envelope) gene encodes a precursor polyprotein, which is cleaved to produce two subunits: a surface gp70 protein and a membrane-anchored p15E protein. The expression of gp70 is modulated during embryonic development and is linked to the differentiation state of the cells [2]. Indeed, retroviral gp70 is a constituent of the surface of various epithelia, thymocytes and peripheral lymphocytes [2–5] and also secreted by hepatocytes as a free protein into the circulation [6]. Significantly, only lupus-prone (NZB $\times$ NZW)F1, MRL and BXSB mice spontaneously develop autoantibodies against serum gp70, detected as gp70-anti-gp70 immune complexes (gp70 IC), and display deposits of these IC in diseased glomeruli [7–9]. A remarkable correlation of serum levels of gp70 IC with the development of severe lupus nephritis underlines the pathogenic role of gp70 IC in murine SLE [10–13].

Serum gp70 has been considered to be a product of xenotropic, PT and mPT retroviruses [14, 15]. Its concentrations are highly variable among different strains of mice [7–9] and largely regulated by the *Sgp3* (serum gp70 production 3) locus, which was identified in lupus-prone mice and controls the transcription of xenotropic, PT and mPT proviruses in livers [15–18]. Moreover, gp70 expression in lupus-prone mice is enhanced by inducers of acute phase proteins, including TLR7 and TLR9 agonists, but unlike conventional acute phase proteins, the acute phase expression of serum gp70 is regulated by the *Sgp3* locus [6, 15, 17, 19].

Analysis of the abundance of different retroviral gp70 RNAs demonstrated that the expression level of mPT gp70 RNAs was selectively increased in lupus-prone mice, as compared with other strains of mice which are not predisposed to autoimmune diseases [20]. Furthermore, many strains of mice expressed not only low levels of the intact wild-type (WT) form of mPT *env* transcripts but also two different *env* deletion mutants, designated D1 and D2, while lupus-prone mice expressed predominantly the WT mPT *env* RNA at the near exclusion of the defective transcripts. This specific expression pattern was regulated by the *Sgp3* locus which is responsible for the more than 100-fold increased transcription of mPT proviruses bearing the intact *env* sequence in lupus-prone mice, as compared with non-autoimmune strains of mice [20].

We have previously shown that the production of gp70 IC implicated in murine lupus nephritis was dependent on the single-stranded RNA-specific innate receptor TLR7 [20, 21] and that the *Sgp3* locus contributed to the development of anti-gp70 autoimmune responses [18]. Thus, *Sgp3* may enhance the production of endogenous retroviral virions carrying single-stranded RNA, which would then promote the development of autoimmune responses against serum gp70 through the activation of TLR7. Since the *Sgp3* locus is responsible for the abundant and predominant expression of the WT mPT *env* transcripts in lupus-prone mice, we investigated the possibility that *Sgp3* is able to induce or enhance the expression of a unique subpopulation(s) of mPT viruses with a pathogenic potential among a number of endogenous mPT proviruses present in the mouse genome. To address this question, we conducted a clonal analysis of mPT viral sequences expressed in C57BL/6 (B6) mice and those congenic for the *Sgp3* locus derived from lupus-prone mice to determine the genetic origin of mPT viral sequences expressed in them. Results obtained from the present study indicate that only 3 of the 13 mPT proviruses present in the B6 genome are actively transcribed in WT mice, likely due to their unique localization within the host genes. In contrast, *Sgp3* congenic mice transcribed multiple but select mPT proviruses, including potentially replication-competent mPT viruses, and stimulation of TLR7 led to a highly enhanced transcription of one potentially replication-competent mPT virus.

2. Materials and methods

2.1 Mice

B6 and C57BL/10 (B10) mice congenic for the NZB- and BXSb-derived *Sgp3* locus (B6.*Sgp3* and B10.*Sgp3*), respectively, were generated by backcross procedures, as described [16, 18]. All studies presented were carried out in 2–3 mo-old female mice. Animal studies described in the present study have been approved by the Ethics Committee for Animal Experimentation of the Faculty of Medicine, University of Geneva (authorization number: 31.1.1005/3049/2-R).

2.2. RT-PCR cloning

RNA from livers was purified with TRIzol reagent (Invitrogen AG, Basel, Switzerland) and treated with DNase I (Amersham Biosciences Corp., Piscataway, NJ). mPT viral cDNA spanning the *env* gene and the U3 region of the long terminal repeat (LTR) was amplified with mPT858F forward primer and Unilr-4R reverse primer, as described [20]. The amplified fragments were purified and ligated into the pBluescript-SK⁺ plasmid. Clones containing the WT form of mPT *env* transcripts were selected by PCR with the use of mPT1115F (5'-GTTCCCAAAACCCATCAGGC-3') and mPT1585R (5'-ATCTAATCCTCTCCGGTTCT-3') primers, which give different sizes of amplicons (471, 355 and 402 bps for WT, D1 mutant and D2 mutant, respectively; Figure 1A). To enrich the intact WT form of mPT amplicons in livers of B6 mice, in which D2 transcripts are predominant [20], amplified fragments derived from WT and D1 mutant were digested with BglI, which is unable to cleave D2 transcripts into two fragments, and cloned into the pBluescript-SK⁺ plasmid.

2.3. Quantitative real-time PCR

The abundance of xenotropic, mPT and PT *env* RNAs (genomic RNA and mRNA) was quantified by real-time PCR, as described [15, 22]. Levels of *cathepsin E* (*Ctse*), *arginine/serine-rich coiled-coil 1* (*Rsrc1*), *UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylglucosaminyltransferase 11* (*Galnt11*), *ADP-ribosylation factor 6* (*Arf6*) and *methyltransferase like 21D* (*Mettl21d*) mRNAs were quantified with the following primers: *Ctse* forward primer (5'-GCCCAACCCATCCACTTAC-3') and reverse primer (5'-TCTCCCTTCCACCCTTCTC-3'); *Rsrc1* forward primer (5'-GCCACCCTGGTAGAACAAGTC-3') and reverse primer (5'-GCACTTCACTTGGTTCTACTGC-3'); *Galnt11* forward primer (5'-GTGGATGACAGCAGTGACTTTG-3') and reverse primer (5'-TCCCTCGCGCTTCATATTTC-3'); *Arf6* forward primer (5'-CCAATCGGTGACCACCATCC-3') and reverse primer (5'-GGTCCCGGTGTAGTAATGCC-3'); *Mettl21d* forward primer (5'-CTCCTGCAGCTAGACTTTGAC-3') and reverse primer (5'-CTTGACCTTACCCAGGATGTTG-3'). PCR was performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Philadelphia, PA) and iQ SYBR green Supermix (Bio-Rad). Results were quantified using a standard curve generated with serial dilutions of a reference cDNA preparation from NZB livers and normalized using TATA-binding protein (TBP) mRNA.

2.4. Genomic PCR

The presence of the *Mpmv11* provirus in mice was determined by genomic PCR analysis with combinations of primers for the U3 sequence and those for *Mpmv11* 5'- and 3'-flanking sequences: U3 forward (5'-AAGCTAGCTGCAGTAACGCCATTTTGC-3') and reverse (5'-AGCTTGCTAAGCCTTATGGTGG-3') primers; *Mpmv11* 5'-flanking forward

(5'-CTATGCTTAGCAGGCAGGTATC-3') and 3'-flanking reverse (5'-AAGGTTGTCTGGCTTGAAGG-3') primers.

2.5. Injection of TLR7 agonist

The TLR7 agonist 1V136 (TLR7 Ligand II, Calbiochem, EMD Chemicals Inc., Darmstadt, Germany) [23] was diluted with sterile PBS and i.p injected into mice. Livers were collected 9 h after injection of 1V136 (100 µg).

2.6. Statistical analysis

Unpaired comparison for frequencies of clones expressing different *Mpmv env* RNAs and for levels of different mRNAs and retroviral gp70 RNAs was analyzed by Student's *t* test. Probability values <5% were considered significant.

3. Results

3.1. The presence of 13 mPT proviruses in the B6 mouse genome

Previous restriction fragment length polymorphism (RFLP) analysis with the use of an mPT-specific *env* probe showed the presence and chromosomal localization of 13 mPT proviral *Mpmv* loci in the B6 mouse genome [24]. This was confirmed by BLAST search analysis, according to gp70 sequences specific for mPT viruses (Table 1). The *Mpmv10* and *Mpmv13* proviruses correspond to D1 and D2 mutants which contain a deletion in the *env* sequence of the 3' portion of the gp70 protein and the 5' portion of the p15E protein, respectively [20], while 11 other mPT proviruses carry the full-length WT form of *env* sequence. The inspection of the coding sequences of the *gag*, *pol* and *env* genes indicated that 6 proviruses (*Mpmv1*, *Mpmv2*, *Mpmv4*, *Mpmv7*, *Mpmv9* and *Mpmv12*) are potentially replication-competent (Table 1). According to the classification reported by Tomonaga *et al.* [25], the U3 structure of LTR of all 13 mPT proviruses is assigned to the P-II subgroup characteristics of mPT viruses (Fig. S1). NZB mice carrying 10 mPT proviruses detected by RFLP analysis [26] share 6 *Mpmv* loci (*Mpmv3*, *Mpmv6*, *Mpmv8*, *Mpmv9*, *Mpmv10* and *Mpmv11*) with B6 mice.

3.2. Predominant transcription of the *Mpmv13* provirus in livers of B6 mice

Our previous RT-PCR analysis of RNA from livers of B6 mice revealed the presence of full-length mPT *env* RNAs as well as two *env* deletion mutants, *Mpmv10* (D1) and *Mpmv13* (D2) [20]. To determine the heterogeneity of the full-length WT mPT *env* transcripts, we amplified, cloned, selected clones containing the full-length *env* transcripts by PCR (Fig. 1A), and sequenced a region spanning the *env* gene and the U3 of the LTR. The nucleotide sequences in this region are highly heterogeneous (Fig. S2) and allow the precise determination of the genetic origin of the transcripts. We observed that livers of B6 mice expressed, in addition to *Mpmv10* (D1) and *Mpmv13* (D2), only a single full-length mPT provirus, *Mpmv6*. Quantitative analysis of ~30 clones from each of three individual mice revealed that, as expected, the expression of *Mpmv13* (D2) was most frequent (mean ± SEM: 72.2 ± 1.8%), followed by that of *Mpmv10* (D1) (15.3 ± 3.1%) and *Mpmv6* (12.5 ± 3.8%; Fig. 1B). Since the number of WT mPT clones analyzed was limited, we enriched WT mPT amplicons by treating them with BglII, which facilitates the purification of digested WT- and *Mpmv10* (D1)-derived fragments. Analysis of additionally obtained 7–10 WT mPT clones from each of three individual mice confirmed that they were all derived from the *Mpmv6* provirus.

The transcription of mPT viruses in livers of B6 mice could be influenced by the regulatory sequences present in the U3 region. However, this is not the case for the selective expression of *Mpmv6*, *Mpmv10* and *Mpmv13* in B6 mice, as the U3 nucleotide sequence of *Mpmv13* is

identical to that of *Mpmv1* and *Mpmv11*, which are not actively transcribed in B6 mice (Fig. S1 and S2). Studies reported that the tRNA primer-binding site sequence can trigger silencing of integrated infectious retroviruses in embryonic cells [27–29]. However, such a mechanism is also excluded for the selective expression of *Mpmv6*, *Mpmv10* and *Mpmv13*, since the nucleotide sequences of the tRNA primer-binding site are different from each other and are themselves shared with other mPT viruses (Fig. S3).

3.3. Predominant transcription of the *Mpmv6* provirus in thymi of B6 mice

Selective transcription of *Mpmv6*, *Mpmv10* (D1) and *Mpmv13* (D2) in B6 mice could be related to their unique integration sites. Indeed, these three proviruses are integrated in the same transcription direction as the host gene within the first intron of the *Ctse* gene, the 4th intron of the *Rsrc1* gene and the 1st intron of the *Galnt11* gene, respectively (Table 1). Thus, we hypothesized that the transcription of these three proviral sequences is favored by their local chromatin environment due to the expression of the corresponding host genes and that the expression levels of these genes in distinct tissues may differ. If so, the expression pattern of these three mPT proviruses may reflect the expression levels of the genes in which they are nested. In this regard, the *Ctse* gene has been reported to be highly expressed in lymphocytes [30] which is in agreement with our finding that the abundance of *Ctse* mRNA was approximately 10-fold higher in thymi than in livers (Table 2). In contrast, the levels of *Rsrc1* and *Galnt11* mRNAs were 3 times less in thymi than in livers ($P < 0.0001$). Analysis of the expression pattern of the three different species of mPT *env* transcripts by mPT-specific RT-PCR revealed the predominant abundance of the full-length form of mPT *env* RNA in thymi, which contrasted with that of the *Mpmv13* (D2) *env* RNA in livers (Fig. 1C). Furthermore, clonal analysis of mPT sequences expressed in the thymi confirmed that the full-length mPT transcript was derived from the *Mpmv6* provirus located within the *Ctse* gene, and its frequency was highest (mean of 4 mice $\pm$ SEM: $80.3 \pm 4.3\%$), followed by that of *Mpmv13* ($16.0 \pm 3.7\%$) and *Mpmv10* ($4.9 \pm 1.9\%$; Fig. 1D). Notably, we were unable to detect transcripts of the *Mpmv2*, *Mpmv8* and *Mpmv9* proviruses, which are integrated in the opposite transcription direction within their respective host genes (Table 1), despite active transcription of host genes (*Abr* for *Mpmv2* and *Fam73a* for *Mpmv9*) in livers and thymi (data not shown). This could be due to possible steric hindrance or interference caused by annealing of the complementary RNAs of the host genes, leading to degradation of corresponding *Mpmv* transcripts [31].

3.4. Predominant transcription of the *Mpmv11* provirus induced by the presence of the *Sgp3* locus in livers of B6 mice

Previous analyses in livers of B6.*Sgp3* congenic mice indicated that the *Sgp3* locus derived from lupus-prone mice selectively upregulates the level of the full-length mPT *env* RNA [20]. Therefore, we investigated whether the upregulation by *Sgp3* derived from lupus-prone NZB mice resulted in the enhanced transcription of the *Mpmv6* provirus or the expression of other mPT proviruses carrying the intact *env* gene. Clonal analysis of mPT sequences expressed in livers of B6.*Sgp3* mice revealed the presence of transcripts derived from not only *Mpmv6*, *Mpmv10* (D1) and *Mpmv13* (D2) but also from multiple other proviruses (Fig. 2A). Strikingly, *Mpmv11* was the most highly expressed provirus, as its frequency reached $62.0 \pm 5.3\%$ (mean of 5 mice $\pm$ SEM). Accordingly, the relative expression frequencies of *Mpmv6*, *Mpmv10* (D1) and *Mpmv13* (D2) were significantly reduced in B6.*Sgp3* mice (*Mpmv6*: $3.4 \pm 1.4\%$; *Mpmv10*: $5.6 \pm 2.5\%$; *Mpmv13*: $6.7 \pm 2.1\%$; $P < 0.05$, $P < 0.05$ and $P < 0.0001$, respectively), as compared with those in WT B6 mice (Fig. 1B). Notably, the previous quantitative PCR analysis showed the lack of increased expression of *Mpmv10* (D1) and *Mpmv13* (D2) in B6.*Sgp3* mice [20]. Collectively, these results suggest that *Sgp3* did not upregulate the expression of the three mPT proviruses expressed in B6 mice. In addition, it was noted that the expression of potentially replication-competent mPT

viruses such as *Mpmv4*, *Mpmv7* and *Mpmv9* was induced by *Sgp3*, although their frequencies were low (Fig. 2A).

The predominant transcription of *Mpmv11* in livers of B6.*Sgp3* mice cannot be attributed to its U3 regulatory sequence as it is identical to that of *Mpmv1* and *Mpmv13* (D2) (Fig. S1 and S2). We explored the possibility that the highly predominant expression of *Mpmv11* in B6.*Sgp3* mice was promoted by enhancer elements of host genes flanking this provirus. However, this possibility appears unlikely because of the lack of upregulated expression in livers of B6.*Sgp3* mice of two neighboring genes, *Arf6* and *Mettl21d*, located ~170 and ~22 Kb upstream and downstream of *Mpmv11* (mean of 5 mice $\pm$ SEM: *Arf6* mRNA; B6.*Sgp3*: 0.9 ± 0.1 , B6: 1.0 ± 0.2 ; *Mettl21d* mRNA; B6.*Sgp3*: 1.4 ± 0.2 , B6: 1.0 ± 0.2).

The *Sgp3* allele of lupus-prone BXSB mice is also responsible for the predominant and abundant expression of full-length mPT *env* transcripts in livers, as demonstrated by the analysis of B10.*Sgp3* congenic mice [20]. Notably, the analysis of B10 mice bearing the *Sgp3* locus derived from BXSB mice confirmed the selectively enhanced expression of *Mpmv11* in their livers (mean of 3 mice $\pm$ SEM: $60.3 \pm 6.5\%$; Fig. 2B), as in the case of B6 mice bearing the NZB-derived *Sgp3* locus (Fig. 2A). However, to our surprise, livers of SB/Le mice failed to display the predominant expression of full-length mPT *env* RNAs (Fig. 2C), despite the fact that the BXSB-*Sgp3* allele is inherited from SB/Le mice (BXSB is a recombinant strain derived from a cross of B6 and SB/Le mice). Thus, the lack of predominant expression of full-length mPT *env* transcripts in livers of SB/Le mice could be due to the absence of the *Sgp3*-responsive *Mpmv11* provirus in the SB/Le genome. This was indeed confirmed by genomic PCR analysis using primers for the U3 region coupled with primers for *Mpmv11* 5'- and 3'-flanking sequences (Fig. 2D).

3.5. Differential effect of *Sgp3* on the transcription of mPT proviruses in thymi and livers of B6 mice

Gv1 (*Gross virus antigen 1*) which is known to control the transcription of endogenous retroviral sequences in different tissues, including the thymus and liver [32], directly overlaps with the *Sgp3* locus [16–18, 33]. However, it has been shown that the *Gv1* locus regulated the transcription of PT proviruses, but not mPT proviruses in thymus [33], while *Sgp3* controlled the transcription of both PT and mPT proviruses in livers [15]. Therefore, we compared the abundance of PT, mPT and xenotropic gp70 RNAs in thymi and livers between B6 and B6.*Sgp3* mice. B6.*Sgp3* mice displayed 6.1- and 9.6-fold increased levels of PT ($P < 0.005$) and xenotropic ($P < 0.001$) gp70 RNAs, respectively, but only a minimal (1.6-fold) increase of mPT ($P < 0.01$) gp70 RNA in thymi, as compared with B6 mice (Fig. 3A). In contrast, *Sgp3* promoted high levels of transcription of all three retroviral sequences in livers: 4.3-, 12.9- and 7.9-fold increases in PT ($P < 0.005$), mPT ($P < 0.0001$) and xenotropic ($P < 0.001$) gp70 RNAs, respectively. Despite the minimal enhancing effect of *Sgp3* on the expression of mPT viruses in thymi, clonal analysis of mPT transcripts revealed a heterogeneous expression profile of mPT proviruses in thymi of B6.*Sgp3* mice, though the frequency of *Mpmv6* remained the highest (mean of 4 mice $\pm$ SEM: $48.9 \pm 6.3\%$), followed by that of *Mpmv11* ($19.1 \pm 3.5\%$; Fig. 3B).

3.6. Enhanced transcription of the potentially replication-competent *Mpmv4* provirus in livers of B6. *Sgp3* mice after stimulation of TLR7

Injection of TLR7 or TLR9 agonists, in addition to LPS (TLR4 agonist), induced the acute phase expression of serum gp70 in B6.*Sgp3* but not B6 mice [15, 17, 19, 34]. Thus, we investigated whether injection of 1V136 (TLR7 agonist) could alter the expression profile of *Mpmv* proviruses in B6.*Sgp3* mice. B6.*Sgp3* mice displayed a 2-fold upregulation of mPT gp70 RNA levels in livers 9h after injection of 1V136 ($P < 0.05$; Fig. 4A). Although no

induced expression of additional *Mpmv* proviruses was observed, the expression pattern of *Mpmv* proviruses in B6.*Sgp3* mice changed after injection of 1V136: the frequency of the potentially replication-competent *Mpmv4* transcript was markedly enhanced, compared with that of control B6.*Sgp3* mice (mean of 4 mice $\pm$ SEM: $45.6 \pm 1.5\%$ vs. $4.9 \pm 2.3\%$; $P < 0.0001$; Fig. 2A and 4B). Notably, the expression profile of *Mpmv* proviruses was not changed in 1V136-injected B6 mice (data not shown).

4. Discussion

Endogenous retroviruses are implicated in the pathogenesis of murine SLE and there is remarkable high-level expression of the mPT retrovirus which is promoted by the *Sgp3* locus in lupus-prone mice [20]. We attempted in the present study to define the specific mPT proviruses expressed in B6 mice carrying or not the *Sgp3* locus derived from lupus-prone mice. The clonal analysis of mPT transcripts revealed the transcription of only 3 of the 13 mPT proviruses present in the B6 genome, possibly as a result of co-regulated expression with the three host genes in which these mPT proviruses are integrated in the same transcription direction. In contrast, we observed that *Sgp3* induced the transcription of distinct and selective subpopulations of mPT proviruses, including proviruses which are potentially replication-competent. Finally, a highly enhanced transcription of a potentially replication-competent mPT virus was detected in B6.*Sgp3* mice after stimulation of TLR7. These observations further highlight the role of *Sgp3* in the pathogenesis of SLE by promoting the generation of potentially infectious retroviruses which could act as a triggering factor for the development of autoimmune responses via TLR7.

Among 13 *Mpmv* proviruses present in the B6 genome, the expression of only *Mpmv6*, *Mpmv10* and *Mpmv13* was detected in B6 mice. Their unique integration in the same transcription direction within the introns of three host genes and the correlation of levels of these three mPT RNAs with those of the host genes between livers and thymi support the idea that these *Mpmv* proviruses are actively transcribed as a result of co-regulated expression with their corresponding host genes. In contrast, *Mpmv* proviruses integrated in the opposite transcription direction within the host genes are not expressed despite transcription of host genes in B6 mice. However, this is clearly not a general mechanism for the transcription of other endogenous retroviruses. Indeed, only two xenotropic viruses (*Xmv10* and *Xmv14*) are expressed in livers among 14 *Xmv* proviruses present in B6 mice, in which *Xmv10* is integrated in the opposite transcription direction within the intron of the actively transcribed host gene (*4921517L17Rik*) encoding a hypothetical protein, LOC70873, and *Xmv14* is not integrated within the host genes (34).

It is intriguing that the effect of the *Sgp3* locus is selective for subpopulations of mPT proviruses as it neither detectably induced the transcription of *Mpmv1*, *Mpmv2*, *Mpmv3* and *Mpmv12* nor upregulated the transcription of the three mPT viruses expressed in WT B6 mice. In addition, among the multiple mPT proviruses newly expressed in B6.*Sgp3* mice, the transcription of one mPT provirus, *Mpmv11*, was more selectively induced by *Sgp3*. This was not due to its U3 regulatory sequence or an *Sgp3*-induced enhanced expression of its neighboring genes. Notably, we have also recently observed that *Sgp3* induced the transcription of only 3 of the 14 xenotropic proviruses in B6 mice [34]. Taken together, these data suggest the presence of a unique genetic mechanism responsible for the selective expression of subpopulations of endogenous retroviruses by *Sgp3*.

The *Sgp3* locus has been narrowed down to an interval between 64.5 and 70.0 Mb of chromosome 13 (unpublished data), in which a cluster of 21 KRAB (Krüppel-associated box)-ZFPs (zinc finger protein) has been identified [35, 36]. ZFPs have been reported to influence the expression of endogenous retroviruses [29, 37, 38]. Notably, ZFP809

suppresses the transcription of a retroviral gene through recognition of a point mutation in the tRNA primer-binding site [29], indicating that a subtle difference, even a single nucleotide polymorphism, in the retroviral sequence could be the target of different KRAB-ZFPs. Moreover, we observed differential effects of *Sgp3* on the transcription of mPT, but not PT and xenotropic viruses, between livers and thymi. These data strongly suggest that several of the *Zfp* genes present in the *Sgp3* locus could be involved in the regulated expression of distinct classes of endogenous retroviruses and even subpopulations of the same class of retroviruses.

Our previous and present analyses raised the possibility that the *Sgp3* locus could induce the generation of replication-competent xenotropic and mPT retroviruses [34]. Although the polymorphic form of the xenotropic and polytropic retrovirus receptor (XPR1) expressed in most laboratory strains of mice does not confer susceptibility to xenotropic viruses, mPT retroviruses are able to utilize XPR1 for infectious entry [39]. In addition, the remarkably enhanced expression of mPT *env* RNA in lupus-prone mice could lead to the generation of chimeric virions containing mPT gp70 proteins and xenotropic genomes [40]. Consequently, such infectious retroviruses could enter plasmacytoid dendritic cells and the activation of these cells through TLR7 could play a substantial role in the accelerated development of SLE through the secretion of IFN α , a cytokine prominently involved in the pathogenesis of SLE [41, 42]. Such a mechanism could account for the spontaneous formation of anti-DNA autoantibodies in *Sgp3* congenic mice [17, 18] and for the development of autoimmune responses against nuclear autoantigens in mice infected with murine leukemia virus [43]. It should also be stressed that infectious retroviruses are not necessarily required for the activation of gp70-specific B cells, since these B cells could be activated through TLR7 following internalization of endogenous retroviruses recognized by the BCR. Thus, the high-level production of non-infectious endogenous retroviruses by *Sgp3* could be sufficient to trigger anti-gp70 autoimmune responses in mice predisposed to SLE.

It is significant that the stimulation of TLR7 enhanced the production of serum gp70 as well as potentially replication-competent retroviruses in B6.*Sgp3* mice as a result of increased secretion of IL-6 and TNF by activated monocytes/macrophages [19, 34]. In addition, the activation of TLR7 induced the development in lupus-prone mice of monocytopia with expansion of a hyperactive monocyte subset in response to IgG IC [21, 44, 45]. Thus, during the course of SLE, an excessive activation of monocytes/macrophages by IgG Fc receptors interacting with RNA-containing IgG IC and then by TLR7 may promote the production of serum gp70 and endogenous retroviruses, thereby providing an additional source for antigenic stimulation and nephritogenic IC formation. Notably, elevated levels of serum gp70 were associated with increased production of gp70 IC and accelerated development of lupus nephritis [46].

Collectively, our data indicate multiple roles of TLR7 in the pathogenesis of SLE. Isolation of replication-competent mPT viruses from lupus-prone mice and assessment of their potential pathogenic activity in mice predisposed to SLE would help define to which extent endogenous retroviruses indeed contribute to the development of SLE. Moreover, the identification of the *Sgp3* gene(s) would help elucidate the molecular basis responsible for the expression of endogenous retroviruses implicated in murine SLE. This would enable us to address the relevance of their human counterparts, thereby providing a clue for the potential role of endogenous retroviruses in human SLE.

Highlights

- Lupus-prone mice express extremely high levels of modified polytropic retroviruses.

- Their expression is controlled by the *Sgp3* locus derived from lupus-prone mice.
- *Sgp3* promotes the expression of unique subpopulations of modified polytropic viruses.
- Replication-competent retroviruses can be highly induced through TLR7 activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Swiss National Foundation for Scientific Research (grant 310030_127644). L.H.E. was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health. We thank Mr Guy Brighouse and Ms Montserrat Alvarez for their excellent technical assistance and Drs Dominique Belin and Thomas Moll for critically reading the manuscript.

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Fig. 1. Expression of different *Mpmv* proviruses in livers and thymi of B6 mice

(A) A region spanning the mPT *env* gene and the U3 region of mPT viral cDNA derived from livers and thymi was amplified and cloned. Clones containing the full-length *env* transcripts (WT) were distinguished from those of deletion mutants, *Mpmv10* (D1) and *Mpmv13* (D2), by PCR analysis with mPT1115F and mPT1585R primers.

(B) and (D) Frequencies of different *Mpmv* transcripts in livers and thymi of B6 mice are shown. Nucleotide sequence of clones containing the full-length mPT *env* transcripts, selected from ~30 (for livers) and ~20 (for thymi) clones in each of 3–4 individual mice, was analyzed to identify the origin of *Mpmv* proviruses carrying the full-length *env*

sequence. Mean percentages (+ SEM) of each *Mpmv* provirus expressed in livers (**B**) and thymi (**D**) are shown.

(**C**) Relative expression pattern of three different species of mPT *env* RNAs in thymi (**Thy**) and livers (**Liv**) was determined by RT-PCR with mPT858F and mPT1585R primers.

Representative results of three individual animals are shown. As a control (**Ctl**), a mixture of three different plasmids containing WT, D1 and D2 clones was included.

Fig. 2. Expression of different *Mpmv* proviruses in livers of C57BL mice congenic for the *Sgp3* locus derived from lupus-prone mice

(A) and (B) Frequencies of different *Mpmv* transcripts in B6.*Sgp3* (A) and B10.*Sgp3* (B) mice congenic for NZB- and BXSB-derived *Sgp3* locus. ~20 clones from each of five B6.*Sgp3* and three B10.*Sgp3* mice were analyzed, and mean percentages (+ SEM) of each *Mpmv* provirus expressed in livers are shown. Potentially replication-competent mPT viruses are indicated by asterisks.

(C) Relative expression pattern of three different species of mPT *env* RNAs in livers of BXSB, B6, SB/Le, B10 and B10.*Sgp3* mice was determined by RT-PCR. Representative

results of three individual animals are shown. Lane 1, BXSB; lane 2, B6; lane 3, SB/Le; lane 4, B10; lane 5, B10.*Sgp3*.

(D) The absence of the *Mpmv11* provirus in SB/Le mice, as determined by genomic PCR analysis. PCR products obtained with U3 forward and *Mpmv11* 3'-flanking reverse primers: lane 1, SB/Le; lane 2, BXSB; lane 3, B6. PCR products with U3 reverse and *Mpmv11* 5'-flanking forward primers: lane 4, SB/Le; lane 5, BXSB; lane 6, B6. PCR products with *Mpmv11* 5'- and 3'-flanking primers: lane 7, SB/Le; lane 8, BXSB; lane 9, B6.

Fig. 3. Levels of PT, mPT and xenotropic gp70 RNAs in thymi and livers of B6.*Sgp3* and WT B6 mice as well as frequencies of different *Mpmv* transcripts in thymi of B6.*Sgp3* mice
(A) Levels of different retroviral gp70 RNAs in thymi (closed column) and livers (open column) of B6.*Sgp3* and WT B6 mice were quantified relative to a standard curve generated with a reference cDNA preparation and normalized using TBP mRNA. Results are expressed as fold increases relative to WT B6 mice (mean of 5 mice + SEM).
(B) Frequencies of different *Mpmv* transcripts in thymi of B6.*Sgp3* mice are shown. ~20 clones from each of four individual mice were analyzed, and mean percentages (+ SEM) of each *Mpmv* provirus expressed in thymi are shown. Potentially replication-competent mPT viruses are indicated by asterisks.

Fig. 4. Levels of mPT gp70 RNA and frequencies of different *Mpmv* transcripts in livers of B6.*Sgp3* mice injected with 1V136

(A) Levels of mPT gp70 RNA in livers 9 h after an i.p. injection of 100 µg of 1V136 or PBS in WT B6 (open column) and B6.*Sgp3* mice (closed column) were quantified relative to a standard curve generated with a reference cDNA preparation and normalized using TBP mRNA. Results are expressed as fold increases relative to PBS-injected WT B6 mice (mean of 4–5 mice + SEM).

(B) Frequencies of different *Mpmv* transcripts in livers of 1V136-injected B6.*Sgp3* mice are shown. ~20 clones from each of four individual mice were analyzed, and mean percentages

(+ SEM) of each *Mpmv* provirus expressed in livers are shown. Potentially replication-competent mPT viruses are indicated by asterisks.

Table 1

Chromosomal localization of mPT proviruses in B6 mice.

<i>Mpmv</i>	Chromosome	Host gene ^a	Replication competence
<i>Mpmv1</i>	7 (71.3) ^b	–	+
<i>Mpmv2</i>	11 (76.2)	<i>Abr</i>	+
<i>Mpmv3</i>	2 (16.3)	–	–
<i>Mpmv4</i>	11 (86.6)	–	+
<i>Mpmv5</i>	10 (4.6)	–	–
<i>Mpmv6</i>	1 (133.5)	<i>Ctse</i>	–
<i>Mpmv7</i>	5 (43.6)	–	+
<i>Mpmv8</i>	11 (102.9)	<i>Plcd3</i>	–
<i>Mpmv9</i>	3 (151.9)	<i>Fam73a</i>	+
<i>Mpmv10 (D1)</i>	3 (66.8)	<i>Rsrc1</i>	–
<i>Mpmv11</i>	12 (70.7)	–	–
<i>Mpmv12</i>	10 (22.5)	–	+
<i>Mpmv13 (D2)</i>	5 (24.7)	<i>Galnt11</i>	–

^a *Mpmv6*, *Mpmv10* and *Mpmv13* are integrated in the same transcription direction within the introns of *Ctse*, *Rsrc1* and *Galnt11*, respectively, while *Mpmv2*, *Mpmv8* and *Mpmv9* in the opposite transcription direction within the introns of *Abr* (active BCR-related), *Plcd3* (phospholipase C, delta 3) and *Fam73a* (family with sequence similarity 73, member A), respectively.

^b Locations of *Mpmv* loci indicated as Mb (megabases) from the centromere.

Table 2 Relative abundance of *Ctse*, *Rsrc1* and *Galnt11* mRNA in thymus and liver of B6 mice.^a

Tissues	<i>Ctse</i>	<i>Rsrc1</i>	<i>Galnt11</i>
Thymus	10.52 ± 1.20	0.33 ± 0.01	0.34 ± 0.02
Liver	0.99 ± 0.22	0.99 ± 0.04	1.00 ± 0.04

^aLevels of mRNA in thymi and livers of B6 mice (mean of 6 mice ± SEM) were quantified relative to a standard curve generated with serial dilutions of a reference cDNA preparation and normalized using TBP mRNA. Results are expressed as fold increases of each transcript in thymi relative to livers.