The Defect in *Fas* mRNA Expression in MRL/*lpr* Mice Is Associated with Insertion of the Retrotransposon, *ETn*

By Jia-Li Chu, Jorn Drappa, Andrew Parnassa, and Keith B. Elkon

From The Hospital for Special Surgery, Cornell University Medical Center, New York, New York 10021

Summary

Fas is a cell surface protein of the tumor necrosis factor receptor, nerve growth factor receptor, CD40 family, and is involved in the control of lymphocyte apoptosis. A mutation in the *Fas* gene in MRL/*lpr* mice results in massive lymphoproliferation (*lpr*) and accelerated autoimmunity. To further study the nature of this defect, *Fas* mRNA expression was evaluated by reverse transcriptase polymerase chain reaction as well as by Northern blotting. These studies revealed that the wild-type *Fas* message was produced at ∼10-fold lower levels in the *lpr* compared with the ++ substrain of MRL mice. In addition to the wild-type transcript, *lpr* mice also synthesized chimeric transcripts containing an insertion of the early retrotransposon (*ETn*). Molecular cloning and nucleotide sequencing of a *Fas-ETn* chimeric cDNA suggested that the striking reduction in wild-type *Fas* mRNA levels and the presence of aberrant transcripts in MRL/*lpr* mice are most likely explained by the insertion of the *ETn* retrotransposon into an intron of the *Fas* gene and induction of alternative splicing involving the 5' *ETn* long terminal repeat.

M RL/*lpr* and MRL/++ are congenic inbred strains of mice that spontaneously develop an autoimmune disease very similar to SLE (reviewed in reference 1). The *lpr* gene is associated with massive infiltration of lymph nodes by double-negative (CD4⁻,CD8⁻) T cells and with accelerated autoimmunity in MRL mice. A spontaneous mutation has also arisen in CBA/K1 mice, resulting in a strain (CBA/*lpr*ᶜᵍ) that develops lymphoproliferation and autoantibodies (2). The *lpr* phenotype in these two strains of mice has recently been shown to result from abnormalities of the *Fas* gene (3). In MRL/*lpr* mice, impaired *Fas* expression was associated with abnormal restriction fragment patterns of *Fas* genomic DNA, whereas in CBA/*lpr*ᶜᵍ mice, a point mutation in the *Fas* gene (a T→A transition at nucleotide 786) was detected. The *lpr*ᶜᵍ mutation results in a replacement of isoleucine with asparagine in the cytoplasmic region of *Fas*, leading to defective signaling for apoptosis by the mutant protein (3).

The precise abnormality of the *Fas* gene in MRL/*lpr* mice has not been identified. To quantify *Fas* expression in MRL mice, reverse transcriptase (RT)[1]-PCR analysis of *Fas* mRNA in lymphoid tissues was undertaken. These studies revealed that MRL/*lpr* mice produced ∼10-fold lower levels of wild-type *Fas* mRNA as well as aberrantly sized *Fas* transcripts.

Materials and Methods

Mice. MRL/*lpr* (MRL/MpJ-*lpr*/*lpr*), MRL/++ (MRL/MP-+/+), NZB/W F₁, and C3H (C3H/HeSnJ) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred at the Hospital for Special Surgery.

Northern and Southern Blotting. Single-cell suspensions were prepared from the thymus, spleen, lymph nodes, and bone marrow of MRL and C3H mice. Total RNA was isolated by the acid guanidinium method (4) and poly(A)⁺-enriched RNA was obtained by the method of Badley et al. (5) using oligo(dT) cellulose (Collaborative Research, Bedford, MA). RNA was blotted to nylon membranes (Gene Screen; New England Nuclear, Boston, MA) and hybridizations were performed according to the manufacturer's instructions. PCR products were also blotted to nylon membranes and hybridized with DNA or oligonucleotide probes. DNA probes were labeled with [³²P]dCTP by the random primer method (6) and oligonucleotides were biotinylated at the 3' termini using terminal transferase (Tdt) (7). After hybridization with oligonucleotides, blots were developed with the chemiluminescent substrate, AMPPD (Tropix, Bedford, MA), and fluorography. All blots were washed under high stringency conditions.

PCR and DNA Sequencing. cDNA was synthesized from total RNA with Moloney murine leukemia virus (MoMULV) RT and oligo(dT) as described (8). cDNA was amplified by PCR on a thermal cycler (480; Perkin Elmer Corp., Norwalk, CT) with Taq polymerase (Bethesda Research Laboratories) unless indicated otherwise. The primers used for PCR analysis are shown in Table 1. The conditions used for amplification were: denaturation at 94°C for 1 min, annealing at 5°C below the melting temperature (Tm) (calculated as described [9]) for 1 min, and extension at 72°C for 1 min except when indicated. Where appropriate, PCR products

---

[1] *Abbreviations used in this paper: ETn,* early retrotransposon; *RT,* reverse transcriptase.

were subcloned into pGEM3 (Promega Biotec, Madison, WI) and sequenced by the dideoxy chain termination method (10) as described (8).

Quantitative PCR. For all quantitative studies, RNA was pooled from groups of three to four "young" (6–8 wk) or "old" (14–20 wk) mice to avoid individual variation. Extensive preliminary studies revealed that optimal quantitation of *Fas* and GAPDH were obtained by 30 cycles of PCR amplification and cDNA dilutions of 1:10 for *Fas* and 1:320 for GAPDH. After RT-PCR, 10 μl of the DNA from each tube was resolved on a 5% polyacrylamide gel. The gel was stained with ethidium bromide and photographed with a video imager (Appligene, Pleasanton, CA) in the reverse mode so that stained DNA appeared as black bands. The relative amount of DNA was quantified by laser densitometry (Molecular Dynamics, Sunnyvale, CA). A tube containing all of the reagents except the cDNA served as the negative control. In each experiment, a standard curve (established by serial dilution of cDNA from Con A-stimulated normal splenocytes) served as the positive control and the control for interassay variation. Results for each test sample were extrapolated from the standard curve and expressed as arbitrary units corresponding to the integrated volume calculated on the laser densitometer. To control for any variation in the RNA input, the results obtained for *Fas* were expressed as a ratio of the results obtained for GAPDH.

Results

MRL/lpr Mice Produce Abnormal Fas mRNA Transcripts. When RT-PCR was performed with the *Fas* primer pair A (see Table 1) and RNA was isolated from the spleens and thymus of MRL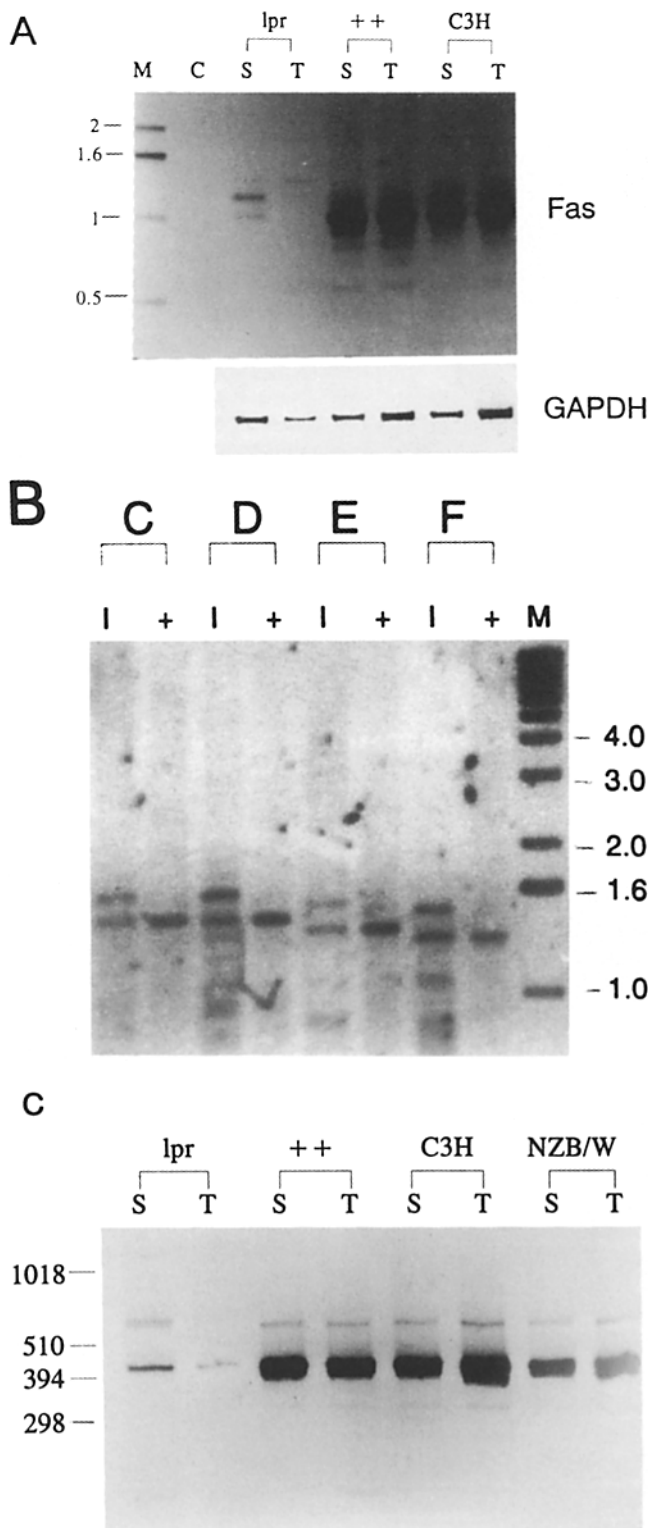/++ and C3H mice, a DNA product of the expected size (1 kb) was observed on ethidium bromide–stained gels (Fig. 1 A). In contrast, a low level of the 1-kb DNA product was amplified after RT-PCR of MRL/lpr RNA, and an additional band ∼200 bp larger than the wild-type PCR product was noted. Similar results were observed when *Fas* primer pair B and either Taq or *Thermophilus litoralis* polymerase (New England Biolabs, Beverly, MA) were used for PCR amplification (not shown), demonstrating that this finding was not a PCR artifact. Since RNase-free DNase (10 μg/ml) had no effect, whereas DNase-free RNase (10 μg/ml) completely eliminated PCR products, the PCR products were derived from RT of RNA rather than contaminating DNA. Although a low level of *Fas* DNA was amplified from *lpr* thymus, this resulted from a lower input of total cDNA as judged by amplification of GAPDH (Fig. 1 A). As shown in Fig. 1 B, when RT-PCR of thymus RNA was performed with four different pairs (Table 1, C–F) and approximately equal amounts of DNA were loaded onto the polyacrylamide gels, DNA products of the expected sizes (wild type) were amplified in both *lpr* and ++ mice, but only *lpr* mice had a second product ∼200 bp larger than the wild type.

To localize the abnormal MRL/lpr *Fas* transcript, RT-PCR was performed with the *Fas* primer pairs G (to amplify the 5' region) and H (to amplify the 3' region). RT-PCR with *Fas* primer pair H amplified DNA products of the expected size (550 bp) in all strains although the amount of MRL/lpr DNA was lower than that derived from the control strains (not shown). RT-PCR with the *Fas* primer pairs G and I revealed that two DNA products were consistently amplified

Table 1. *Oligonucleotides Used for PCR and Hybridization*

	PCR	
Sense		**Antisense**
Fas		
A (50) ATGCTGTGGATCTGGGCTGT		(1067) TCTCCTCTCTTCATGGCTGG
B (29) CTGTTTTCCCTTGCTGCAGACATG		(1200) GTCCCTGGAACCTGCTAGTCATTT
C (29) CTGTTTTCCCTTGCTGCAGACATG		(1480) TGAAATGCAAAAAGAGATAC
D (50) ATGCTGTGGATCTGGGCTGT		(1480) TGAAATGCAAAAAGAGATAC
E (127) CATCTCCGAGAGTTTAAAGC		(1480) TGAAATGCAAAAAGAGATAC
F (185) GAAGGATTATATCAAGGAGGCCCA		(1480) TGAAATGCAAAAAGAGATAC
G (50) ATGCTGTGGATCTGGGCTGT		(539) GTTTCCTGCAGTTTGTATTGCT
H (517) AGCAATACAAACTGCAGGAAAC		(1067) TCTCCTCTCTTCATGGCTGG
I (127) CATCTCCGAGAGTTTAAAGC		(539) GTTTCCTGCAGTTTGTATTGCT
GAPDH		
GA (242) CCATCACCATCTTCCAGGAG		(817) CCTGCTTCACCACCTTCTTG
	Hybridization	
ETn8 (27) CTTCTCAGTCGAACCGTTCACGTT		

Oligonucleotides are shown as 5' → 3', with the base numbers of the most 5' nucleotide given in parentheses.

from all test strains although the larger DNA product represented only a minor fraction of the amplified DNA in the control strains (Fig. 1 C). In addition, the larger PCR product amplified from the control strains was slightly smaller than that obtained from *lpr* mice. RT-PCR of RNA obtained from *lpr* liver and kidney with primer pair G also revealed two DNA products of 500 and 700 bp (not shown).

To verify that the PCR products amplified with *Fas* primers were specific for *Fas*, a Southern blot was performed with a 1-kb *Fas* probe (the PCR product amplified from MRL/+ + thymus with the primer pair A). As shown in Fig. 2, *A* and *B*, both of the *lpr*-derived products amplified from spleen and thymus cDNA hybridized with the 1-kb *Fas* probe, whereas cDNA amplified from + + mice showed a single band corresponding to the PCR product stained with ethidium bromide. An additional product of ∼800 bp also hybridized with the *Fas* probe in *lpr*- but not + + -derived PCR products (Fig. 2 B).

Northern Blot Analysis. Poly(A)⁺-enriched RNAs obtained from MRL/*lpr*, MRL/+ + , and C3H thymus and liver were hybridized with the 1-kb *Fas* probe. As shown in Fig. 3, a transcript of ∼1.8 kb hybridized with this probe in all three strains and the level of *Fas* mRNA expression was considerably lower in *lpr* thymus and liver compared with the control strains. While not distinctly resolved as a separate transcript, the *lpr*-derived *Fas* mRNA appeared to be slightly larger than the *Fas* mRNA in the controls. In addition, the *Fas* probe hybridized with a transcript ∼0.8 kb in *lpr* thymus and liver, but not with RNA derived from the control strains. Weaker hybridization with transcripts of ∼13 kb were also observed in the *lpr*-derived thymus RNA, but similar bands were observed in the control strains after longer exposure of the autoradiograph (not shown). These bands may represent unspliced nuclear *Fas* RNA. A higher background was consistently observed in poly(A)⁺-enriched DNA derived from *lpr* thymus for reasons that are unknown.

Aberrant MRL/lpr Fas mRNA Contains an Insertion of the Early Retrotransposon (ETn). To determine the nature of the DNA products obtained from MRL/*lpr* mice after RT-PCR amplification with *Fas* primer pair G, the two PCR products obtained from *lpr* thymus were subcloned and sequenced. Nucleotide sequencing revealed that the 500-bp PCR product contained the expected sequence reported for *Fas* (11). The 700-bp product contained an insertion of 183 bp at position 233 of *Fas* DNA without altering the translational reading frame of *Fas*. A search of the GenBank and EMBL databases revealed that the 183-bp insertion had a 98.4% sequence iden-

Figure 1. (*A*) RT-PCR of RNA obtained from the spleens (*S*) and thymus (*T*) of MRL/*lpr*(*lpr*), MRL/+ +(+ +), and C3H mice. DNA amplification was performed with *Fas* primer pair A (see Table 1), and 10 μl of the products was resolved on a 5% polyacrylamide gel. The gel was stained with ethidium bromide and photographed with a video imager in the reverse mode (the stained bands appear black). GAPDH was amplified from the same RNA samples using primer pair GA (Table 1). M, size markers (kb); C, control (RT-PCR performed with *Escherichia coli*

tRNA). (*B*) RT-PCR of RNA obtained from the thymus of MRL/*lpr*(*l*) and + +(+) mice with four different primer pairs. DNA amplification was performed with *Fas* primer pairs C and F (shown above), and either 10 μl (*lpr*) or 2 μl (+ +) of the products was applied to a 0.8% agarose gel. After electrophoresis the gel was stained with ethidium bromide as described in *A*. (*C*) RT-PCR of the 5′ *Fas* mRNA. DNA amplification was performed with *Fas* primer pair I and cDNA obtained from the spleen (*S*) and thymus (*T*) of MRL/*lpr*, MRL/+ , C3H, and NZB/W mice. 10 μl of the products was subjected to electrophoresis on a 5% polyacrylamide gel and stained with ethidium bromide. The positions of the size markers (bp) are shown on the left.

B

C D E F

I + I + I + I +

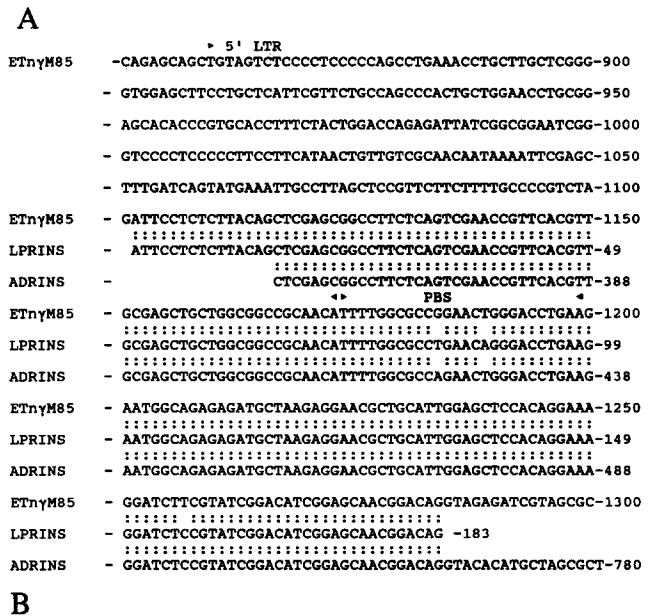1.6 —

1.0 —

A

lpr ++ C3H

M C S T S T S T

2 —
1.6 —
1 —
0.5 —
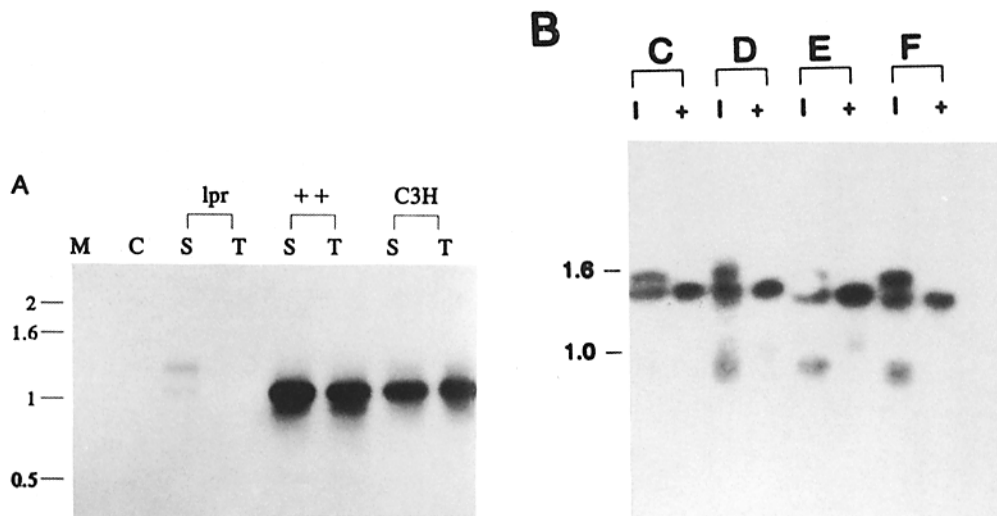
Figure 2. Southern blot analysis with a *Fas* probe. The amplified DNA shown in Fig. 1, *A* and *B*, respectively, was resolved on a 0.8% agarose gel, blotted to nylon membranes, and probed with the ^{32}P-labeled 1-kb *Fas* probe (a RT-PCR product obtained from MRL/+ + thymus with primer pair A). The corresponding Southern blots are shown in *A* and *B*.

tity with a region of the 5' LTR and primer binding site of the *E Tn* retrotransposon inserted into the mouse IgG1 heavy chain switch region in the BALB/c myeloma, P3.26Bu4 (Fig. 4 *A*). The molecular clone isolated from P3.26Bu4 was designated γM85 (12). The 183-bp insert also had 99% sequence identity with the corresponding region of the *E Tn* retrotransposon that has disrupted the skeletal muscle chloride channel in ADR mice (13) (Fig. 4 *A*). In contrast, the *E Tn* insert detected in *lpr Fas* mRNA had only 51% sequence identity with the prototypic *E Tn* sequence, MG1 (14) (Fig. 4 *B*). As shown in Fig. 5, where the nucleotide sequences of *Fas* and *E Tn* are known or presumed, the proposed 5' and 3' splice sites closely resembled the splice consensus sequences (15).
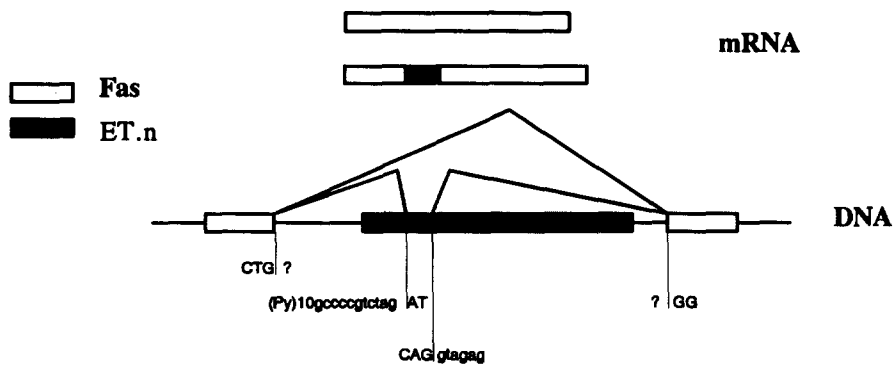
Southern and Northern Blot Analysis of E Tn Expression. To determine whether additional aberrant transcripts containing *E Tn* were amplified from *lpr*-derived cDNA and whether the minor DNA products amplified in the control strains (Fig. 1 *C*) contained *E Tn* sequences, Southern blots with *E Tn* probes were performed. When the Southern blots shown in Fig. 2, *A* and *B*, were stripped and rehybridized with the *E Tn*

A

```
                           • 5' LTR
ETnγM85    -CAGAGCAGCTGTAGTCTCCCCTCCCCCAGCCTGAAACCTGCTTGCTCGGG-900

           - GTGGAGCTTCCTGCTCATTCGTTCTGCCAGCCCACTGCTGGAACCTGCGG-950

           - AGCACACCCGTGCACCTTTCTACTGGACCAGAGATTATCGGCGGAATCGG-1000

           - GTCCCCTCCCCCTTCCTTCATAACTGTTGTCGCAACAATAAAATTCGAGC-1050

           - TTTGATCAGTATGAAATTGCCTTAGCTCCGTTCTTCTTTTGCCCCGTCTA-1100

ETnγM85    - GATTCCTCTCTTACAGCTCGAGCGGCCTTCTCAGTCGAACCGTTCACGTT-1150
             :::::::::::::::::::::::::::::::::::::::::::::::::::
LPRINS     - ATTCCTCTCTTACAGCTCGAGCGGCCTTCTCAGTCGAACCGTTCACGTT-49
             :::::::::::::::::::::::::::::::::::::::::::::
ADRINS     -                 CTCGAGCGGCCTTCTCAGTCGAACCGTTCACGTT-388
                                             ◄►    PBS       ◄
ETnγM85    - GCGAGCTGCTGGCGGCCGCAACATTTTGGCGCCGGAACTGGGACCTGAAG-1200
             ::::::::::::::::::::::::::::::::::::  :::: :::::::::
LPRINS     - GCGAGCTGCTGGCGGCCGCAACATTTTGGCGCCTGAACAGGGACCTGAAG-99
             ::::::::::::::::::::::::::::::::::::  :::: :::::::::
ADRINS     - GCGAGCTGCTGGCGGCCGCAACATTTTGGCGCCAGAACTGGGACCTGAAG-438
ETnγM85    - AATGGCAGAGAGATGCTAAGAGGAACGCTGCATTGGAGCTCCACAGGAAA-1250
             ::::::::::::::::::::::::::::::::::::::::::::::::::::
LPRINS     - AATGGCAGAGAGATGCTAAGAGGAACGCTGCATTGGAGCTCCACAGGAAA-149
             ::::::::::::::::::::::::::::::::::::::::::::::::::::
ADRINS     - AATGGCAGAGAGATGCTAAGAGGAACGCTGCATTGGAGCTCCACAGGAAA-488
ETnγM85    - GGATCTTCGTATCGGACATCGGAGCAACGGACAGGTAGAGATCGTAGCGC-1300
             :::::::  :::::::::::::::::::::::::::::::
LPRINS     - GGATCTCCGTATCGGACATCGGAGCAACGGACAG   -183
             :::::::::::::::::::::::::::::::::::
ADRINS     - GGATCTCCGTATCGGACATCGGAGCAACGGACAGGTACACATGCTAGCGCT-780
```

B

```
ETNMG1     - GCCCCTCTTCTCTTCCAGGTTTCCAAAATGCCTTTCCAGGCTAGAACCCT -50
             ::::::   ::: ::: : :    : : ::          :   :
LPRINS     - ATTCCTCT---CTTACAGCT--CGAGCGGCCTTCTCAGTCGAACCGTTCA -45

ETNMG1     - GGTTGTGGTCTGCTGGCCGGACACAACAATTGGCGAACCAACGCGGGACT -100
             ::::: :  ::::::::: :     :    :::::::    ::: :: :::
LPRINS     - CGTTGCGAGCTGCTGGCGGCCGCAACATTTTGGCGCCTGAACAGGGACCT -95

ETNMG1     - GAGAAACGGCAAAGGATTTTTGGAAGAGACGCTGCTGGTTCGGAGCTCCA -150
             ::  :: :::: ::   :  :  :: ::::::::    : :::::::::
LPRINS     - GAAGAATGGCAGAGAGATGCTAAGAGGAACGCTGCA---TTGGAGCTCCA -142

ETNMG1     - TAAAATAAAGGATAAGGGAAATTCATACCGGAGAAGGTATG -191
             : : :::  :    :      ::: ::: : : :::::
LPRINS     - CAGGAAAGGATCTCCGTATCGGACATCGGAGCAACGGACAG -183
```

Figure 4. Nucleotide sequence comparisons between the *E Tn* insertions detected in MRL/*lpr Fas* mRNA (*LPRINS*), the skeletal muscle chloride channel in ADR mice (*ADRINS*), and the prototype *E Tn* subfamily sequences. Identity between LPRINS and ETnγM85 (12, 16) is 98%, and between LPRINS and ADRINS (13) is 99% (*A*). Identity between LPRINS and ETnMG1 (14) is only 51% (*B*). The positions of the 5' LTR and primer binding site (*PBS*) are shown in *A*. These sequence data have been submitted to GenBank under accession no. L15352.

thymus liver

I + C I + C

28 S —

18 S —

Figure 3. Northern blot analysis of *Fas* expression in thymus and liver. 2 μg of poly(A)+-enriched RNA obtained from *lpr* (*l*), ++(+), or C3H (*C*) mice was applied to each lane of a 1% agarose gel containing 2.2 M formaldehyde. After transfer to a nylon membrane, hybridization was performed with the same ^{32}P-labeled *Fas* probe described in Fig. 2. The positions of the 18 and 28 S RNAs are shown.

mRNA

DNA

Fas
ET.n

CTG ?
(Py)10gccccgtctag AT
CAG gtagag
? GG

Figure 5. Proposed mechanism of the *Fas* mutation in MRL/*lpr* mice. *ETn* inserts into an intron of the *Fas* gene. The insertion induces aberrant splicing of the *Fas* gene and reduced transcription or stability of the spliced messenger RNAs. It is currently unknown whether *ETn* is inserted as a full-length (5.7 kb) or partial retrotransposon. The nucleotide sequences, where known (*Fas* [3] and *ETn*INS [see Fig. 4], shown as uppercase) or presumed (derived from *ETn*γM85 [12, 16], shown as lowercase), are provided at the proposed alternative splice sites. This is not drawn to scale.

probe 41 (a 2-kb Pst fragment of RMg2 containing the 5' LTR and primer binding site homologous to γM85 [16], kindly provided by W. Dunnick, University of Michigan, Ann Arbor, MI), only the product 200 bp larger than wild-type cDNA from *lpr* thymus (Fig. 6 *A*) and spleen (not shown) hybridized. In addition, the ~800-bp product that hybridized with the *Fas* probe also hybridized with the *ETn* probe, indicating that this was also likely to result from a chimeric transcript. When the PCR products shown in Fig. 1 *C* were

rehybridized with the *ETn* oligonucleotide 8 (Table 1), the larger DNA fragment amplified from *lpr*, but not the control, mice hybridized (Fig. 6 *B*). These findings demonstrate that only two *Fas*/*ETn* chimeric products were consistently amplified from MRL/*lpr* cDNA, and that the additional product amplified with the primer pair I in the control strains (Fig. 1 *C*) either represents a minor alternatively spliced form of *Fas* mRNA or is a PCR artifact.

When the Northern blot shown in Fig. 3 was stripped
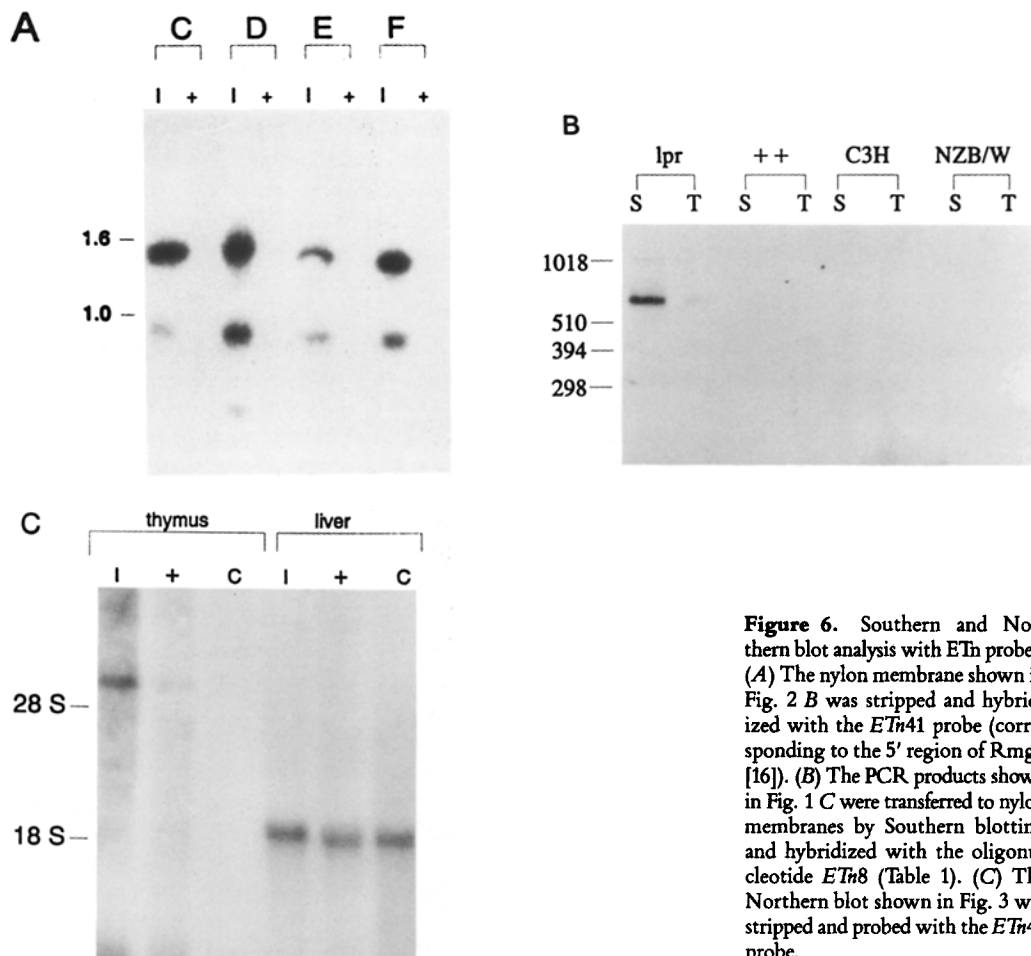
Figure 6. Southern and Northern blot analysis with ETn probes. (*A*) The nylon membrane shown in Fig. 2 *B* was stripped and hybridized with the *ETn*41 probe (corresponding to the 5' region of Rmg2 [16]). (*B*) The PCR products shown in Fig. 1 *C* were transferred to nylon membranes by Southern blotting and hybridized with the oligonucleotide *ETn*8 (Table 1). (*C*) The Northern blot shown in Fig. 3 was stripped and probed with the *ETn*41 probe.

and hybridized with the *ETn* 41 probe (Fig. 6 *C*), the most intense hybridization from thymus RNA was observed at 6 kb, as has previously been reported for lymphoid cell lines and spleen (12). In contrast, a strong hybridization signal was observed at 2 kb from RNA isolated from the liver of all three test strains. Although the size of this band was close to the 1.8-kb *Fas* transcript, it was clearly distinguishable when the autoradiographs were superimposed.

MRL/lpr Mice Produce ~10-fold Lower Levels of Fas mRNA. To confirm that the RT-PCR method used could quantify differences in mRNA input, thymus RNA obtained from MRL/+ + mice was serially diluted, subjected to RT-PCR with primer pair B, and quantified as described in Materials

and Methods. As shown in Fig. 7 *A*, a dose-response curve was observed under these conditions. The relative amounts of *Fas* mRNA amplified with primer pair G after RT-PCR of RNA obtained from bone marrow, spleen, lymph node, and thymus of MRL mice are shown in Fig. 7 *B*. Regardless of the source of RNA, *lpr* mice expressed ~10% of the wild-type *Fas* mRNA compared with the congenic + + strain. Similar results were obtained when PCR was performed with the *Fas* primer pair I (not shown).

Discussion

MRL/*lpr* and MRL/+ + mice are thought to be genetically identical except for a mutation in the *Fas* gene in the *lpr* substrain (1). The present study demonstrates that *lpr* mice have an ~10-fold reduction in the level of *Fas* mRNA and produce aberrant mRNA transcripts containing an insertion of a fragment of the retrotransposon, *ETn*. *ETn* is a moderately repetitive (~200 copies) retrotransposon first identified by its high level of expression in embryos and in teratocarcinoma cells (17). Although the prototypic *ETn* sequence, MG1, isolated from a BALB/c genomic library was originally thought to be highly conserved (14), significant sequence differences have now been identified in the U5 region and immediately downstream of the 5' LTR of many *ETn* retrotransposons that have disrupted cellular genes (12, 13, 16, 18, 19). Since these apparently mobile *ETn* retrotransposons are themselves highly homologous, they are now regarded as a separate subfamily designated *ETn* γM85 (16) (according to the prototype *ETn* retrotransposon inserted into the mouse IgG1 heavy chain switch region in the BALB/c myeloma, P3.26Bu4 [12]). The nucleotide sequence of the 183-bp *ETn* fragment inserted at nucleotide 233 of the MRL/*lpr Fas* cDNA is virtually identical to the corresponding γM85 *ETn* sequence.

In addition to transposition in lymphoid cells, *ETn* insertion has recently been reported to cause myotonia in ADR mice (13). Insertion of *ETn* in an intron of the skeletal muscle chloride channel (*CIC*) gene disrupts the gene by inducing three abnormal *CIC/ETn* chimeric transcripts through alternative splicing (13). RT-PCR and Northern blot analysis of *Fas* expression in MRL/*lpr* mice revealed at least two aberrant *Fas/ETn* chimeric transcripts. Despite the large size of the full-length *ETn* retrotransposon (5.7 kb) (14), the position of the splice site identified in two of the three *CIC/ETn* transcripts is located very close to the origin of the *ETn* insert at nucleotide 233 of *lpr Fas* cDNA. While the proximity is explained, in part, by the polypyrimidine tracts (see Figs. 4 and 5), other sequence or structural features of the U5 region of the *ETn* LTR may facilitate alternative splicing. The splicing pattern of the smaller (~800-bp) *Fas/ETn* transcript has not yet been determined.

Retrotransposon insertion may either increase or decrease expression of a gene (reviewed in reference 20). The 10–20-fold reduction in the expression of *C4* in H-2^k mice is associated with the insertion of the *B2* retrotransposon into intron 13 of the *C4* gene (21). *B2* contains an alternative splice site and produces an abnormally spliced *B2/C4* transcript as well as low levels of the wild-type transcript (21). Transfec-
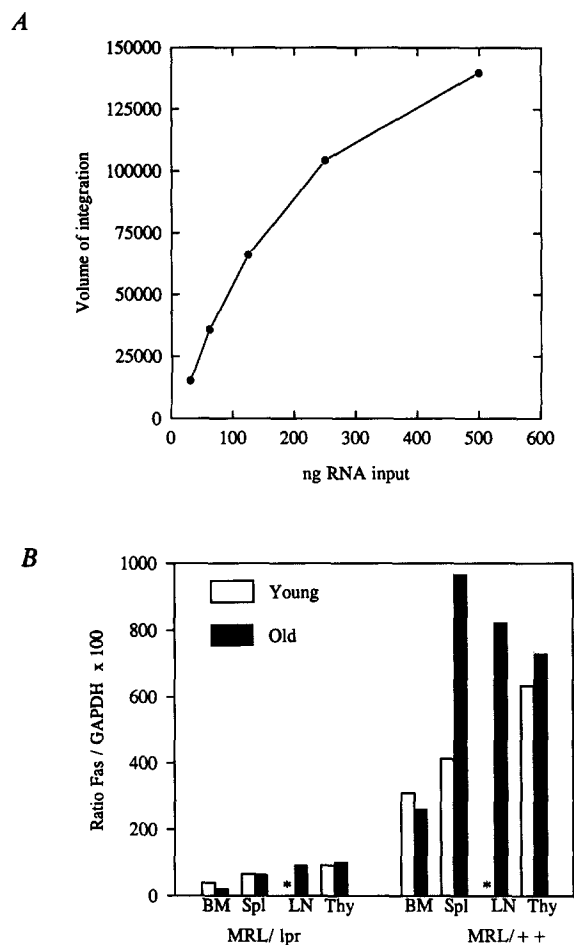
Figure 7. Quantitative analysis of *Fas* mRNA expression in MRL mice. (*A*) MRL/+ + thymus RNA was serially diluted and subjected to RT-PCR with *Fas* primer pair C. After electrophoresis, ethidium bromide staining of the PCR products was quantified by laser densitometry. Results are expressed as the volume of integration. (*B*) Bone marrow (*BM*), spleen (*Spl*), lymph nodes (*LN*), and thymus (*Thy*) were obtained from three "young" (4–6-wk-old) and three "old" (4–5-mo-old) *lpr* and + + mice. RNA was isolated and RT-PCR performed with primer pair G as described in Materials and Methods. The amount of DNA amplified was quantified by video imaging and laser densitometry of ethidium bromide–stained gels, and the number of units was extrapolated from a standard curve. The results are expressed as the ratio of *Fas*/GAPDH × 100. *Not tested.

tion experiments revealed that aberrant splicing of *C4* mRNA was sufficient to cause the profound defect in the expression of *C4* (22). Since expression of a normal-sized *Fas* transcript was detected by RT-PCR and Northern blotting in *lpr* mice, *ETn* is almost certainly present in an intron of the *Fas* gene. We therefore propose that the inserted *ETn* retrotransposon provides alternative splice site(s) and, analogous to the *B2/C4* gene model, reduces *Fas* mRNA levels either through reduced transcription or stability of the message. This interpretation differs from the study of Watson et al. (23), who have recently reported that the *Fas* gene in MRL/*lpr* mice contains a 1.4-kb deletion as determined by restriction fragment analysis and Southern blotting. However, further genomic analysis has shown results that are most consistent with a 5.7-kb insertion in the *Fas* gene in MRL/*lpr* mice (M. L. Watson and M. F. Seldin, personal communication). Attempts to amplify *ETn* from *lpr* genomic DNA by PCR using primers flanking the *ETn* insertion identified in *lpr Fas* cDNA have been unsuccessful due to the limited size that can be amplified from genomic DNA by this technique (J. L. Chu, and K. B. Elkon, unpublished observations). Similarly, the ability to identify a single copy of *ETn* integrated into the MRL/*lpr Fas* gene by Southern blot analysis of genomic DNA is complicated by the presence of ∼200 copies of *ETn* distributed throughout the mouse genome.

It is notable that many of the reported insertions of the *ETn* retrotransposon have been identified in genes of immunological interest (12, 16, 18, 19). Since *ETn* RNA is expressed in lymphoid cell lines and in normal spleen but is not known to encode long open reading frames (12–14), *trans*-acting elements present in lymphoid cells may enhance expression of *ETn*. Interestingly, the size of the *ETn* transcripts derived from thymus and liver differed. Detection of a 2-kb transcript in RNA obtained from the liver of MRL and C3H mice suggests either that the *ETn* is constitutively expressed and the transcript is spliced in the liver, or that the *ETn* LTR is inserted into a normal transcript. Since an *ETn* insertion has been identified in the 3' untranslated region of the *Q6* and *Q8* genes of the mouse MHC (19), this observation is not without precedent. Although the point mutation detected in the *Fas* gene of CBA/*lpr*cg mice indicates that the insertion of *ETn* is not the sole mechanism responsible for altera-

tion of the *Fas* gene, it remains to be determined whether other background abnormalities present in MRL mice (1) facilitate transposition and/or expression of the *ETn* retrotransposon.

The *lpr* gene is responsible for acceleration of lupus in MRL mice and for the production of autoantibodies and/or lymphadenopathy when backcrossed onto normal mouse strains (24–26). While the conclusion by Watanabe-Fukunaga et al. (3) is that the absence of *Fas* expression results in the failure of negative selection in the thymus, the appropriate TCR Vβ deletion in unmanipulated MRL/*lpr* mice (27, 28) implies either that *Fas* does not play a role in Vβ-related negative selection in the thymus, or that the 10-fold reduction in *Fas* expression observed in the present study is sufficient to allow negative selection of these thymocytes to occur. Although the sequence of the full-length *Fas* cDNA from *lpr* mice has not yet been determined, a wild-type product was detected by Northern blot and PCR analyses. In addition, the *ETn* insert detected in the *Fas* mRNA did not alter the translational reading frame. These findings suggest that a small amount of *Fas* protein may be synthesized in *lpr* mice. If the defect in negative selection in the thymus is quantitative rather than absolute, this may explain why negative selection of most T cells in the thymus is apparently normal in MRL/*lpr* mice but is reduced in transgenic *lpr/lpr* H-2D^b HY^+ male mice where all of the thymocytes are autoreactive (29).

Failure to detect *Fas* mRNA expression in the spleens of normal mice by Northern blot analysis (11) suggested that the *Fas* mutations impair negative selection of thymocytes rather than mature T cells (3). In the present study, *Fas* mRNA expression was readily detectable in RNA obtained from C3H control, MRL/+ +, and NZB/W spleen cells by RT-PCR, indicating that *Fas* may play a role in regulating the survival of mature lymphocytes. The increase in the level of expression of *Fas* mRNA observed in the spleens of MRL/+ + mice with age may reflect lymphocyte activation, since *Fas* expression in human lymphocytes has been shown to be upregulated after stimulation (30, 31). While detection of *Fas* mRNA expression in NZB/W thymus and spleen at levels comparable to controls indicates that impaired *Fas* expression is not a universal feature of murine lupus, further studies are required to evaluate the *Fas* protein function in murine and human SLE.

Address correspondence to Keith Elkon, The Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021.

Note Added in Proof: Sequencing of additional *Fas/ETn* chimeric cDNAs after RT-PCR with primer pair I has revealed a transcript with a 168-bp insertion of *ETn* identical to the ADRINS sequence shown in Fig. 4 *A* (GenBank accession no. L15353). After submission of this manuscript, Adachi et al. (32) reported that a 5.7-kb *ETn* retrotransposon has been detected in intron 2 of the *Fas* gene in MRL/*lpr* mice.

References

1. Cohen, P.E., and R.A. Eisenberg. 1991. *lpr* and *gld*: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9:243.

2. Matsuzawa, A., T. Moriyama, T. Kaneko, M. Tanaka, M. Kimura, H. Ikeda, and T. Katagari. 1990. A new allele of the *lpr* locus, *lpr*^{cg}, that complements the *gld* gene in induction of lymphadenopathy in the mouse. *J. Exp. Med.* 171:519.

3. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in *Fas* antigen that mediates apoptosis. *Nature (Lond.).* 356:314.

4. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.

5. Badley, J.E., G.A. Bishop, T. St. John, and J.A. Frelinger. 1988. A single, rapid method for the purification of poly A RNA. *Biotechniques.* 6:114.

6. Feinberg, A.P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease to high specific activity. *Anal. Biochem.* 132:6.

7. Riley, L.K., E.M. Marshall, and M.S. Coleman. 1986. Laboratory methods: A method for biotinylating oligonucleotide probes for use in molecular hybridizations. *DNA (NY).* 5:333.

8. Elkon, K.B., J.J. Hines, J.L. Chu, and A.P. Parnassa. 1990. Epitope mapping of recombinant HeLa SmB and B' peptides obtained by the polymerase chain reaction. *J. Immunol.* 145:636.

9. Freier, S.M., R. Kierzek, J.A. Jaeger, N. Sugimoto, M.H. Caruthers, T. Neilson, and D.H. Turner. 1986. Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci. USA.* 83:9373.

10. Sanger, F., S. Nickleu, and A.R. Coulsen. 1977. DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci. USA.* 54:5463.

11. Watanabe-Fukunaga, R., C.I. Brannan, N. Itoh, S. Yonehara, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. The cDNA structure, expression, and chromosomal assignment of the mouse *Fas* antigen. *J. Immunol.* 148:1274.

12. Shell, B., P. Szurek, and W. Dunnick. 1987. Interruption of two immunoglobulin heavy chain switch regions in a murine plasmacytoma p3.26Bu4 by insertion of retrovirus-like element ETn. *Mol. Cell. Biol.* 7:1364.

13. Steinmeyer, K., R. Klocke, C. Ortland, M. Gronemeier, H. Jockusch, S. Grunder, and T.J. Jentsch. 1991. Inactivation of muscle chloride channel by transposon insertion in myotonic mice. *Nature (Lond.).* 354:304.

14. Sonigo, P., S. Wain-Hobson, L. Bougeleret, P. Toillas, F. Jacob, and P. Brulet. 1987. Nucleotide sequence and evolution of ETn elements. *Proc. Natl. Acad. Sci. USA.* 84:3768.

15. Breathnach, R., and P. Chambon. 1981. Organization and expression of eucaryotic split genes coding for proteins. *Annu. Rev. Biochem.* 50:349.

16. Shell, B.E., J.T. Collins, L.A. Elenich, P.F. Szurek, and W.A. Dunnick. 1990. Two subfamilies of murine retrotransposon ETn sequences. *Gene (Amst.).* 86:269.

17. Brulet, P., M. Kaghad, Y.-S. Xu, O. Croissant, and F. Jacob. 1983. Early differential tissue expression of transposon-like repetitive DNA sequences of the mouse. *Proc. Natl. Acad. Sci. USA.* 80:5641.

18. Weiss, S., and B. Johansson. 1989. Integration of the transposon-like element ETh upstream of Vλ2 in the cell line P3X63Ag8. *J. Immunol.* 143:2384.

19. Elliot, E., D. Rathbun, A. Ramsingh, J. Garberi, and L. Flaherty. 1989. Genetics and expression of the Q6 and Q8 genes: an LTR-like sequence in the 3' untranslated region. *Immunogenetics.* 29:371.

20. Keshet, E., R. Schiff, and A. Itin. 1991. Mouse retrotransposon: A cellular reservoir of long terminal repeat (LTR) elements with diverse transcriptional specificities. *Adv. Cancer Res.* 56:215.

21. Pattanakitsakul, S., J.H. Zheng, S. Natsuume-Sakai, M. Takahashi, M. Nonaka. 1992. Aberrant splicing caused by the insertion of the B2 sequence into an intron of the complement C4 gene is the basis for low C4 production in H-2^k mice. *J. Biol. Chem.* 267:7184.

22. Zheng, J.H., S. Natsuume-Sakai, M. Takahashi, and M. Nonaka. 1992. Insertion of the B2 sequence into intron 13 is the only defect of the H-2^k C4 gene which causes low C4 production. *Nucleic Acids Res.* 20:4975.

23. Watson, M.L., J.K. Rao, G.S. Gilkeson, P. Ruiz, E.M. Eicher, D.S. Pisetsky, A. Matsuzawa, J.M. Rochelle, and M.F. Seldin. 1992. Genetic analysis of MR1/*lpr* mice: Relationship of the *Fas* apoptosis gene to disease manifestations and renal disease-modifying loci. *J. Exp. Med.* 176:1645.

24. Morse, H.C., III, W.F. Davidson, R.A. Yetter, E.D. Murphy, J.B. Roths, and R.L. Coffman. 1982. Abnormalities induced by the mutant gene *lpr*: expansion of a unique lymphocyte subset. *J. Immunol.* 129:2612.

25. Izui, S., V.E. Kelley, K. Masuda, H. Yoshida, J.B. Roths, and E.D. Murphy. 1984. Induction of various autoantibodies by mutant gene *lpr* in several strains of mice. *J. Immunol.* 133:227.

26. Warren, R.F., S.A. Caster, J.B. Roths, E.D. Murphy, and D.S. Pisetsky. 1984. The influence of the *lpr* gene on B cell activation: differential antibody expression in *lpr* congenic mouse strains. *Clin. Immunol. Immunopathol.* 31:65.

27. Kotzin, B.L., S.K. Babcock, and L.R. Herron. 1984. Deletion of potentially self-reactive T cell receptor specificities in L3T4⁻, Lyt 2⁻ T cells of *lpr* mice. *J. Exp. Med.* 168:2221.

28. Singer, P.A., R.S. Balderas, R.J. McEvilly, M. Bobardt, and A.N. Theofilopoulos. 1989. Tolerance-related Vβ clonal deletions in normal CD4⁻8⁻, TCR-α/β⁺ and abnormal *lpr* and *gld* cell populations. *J. Exp. Med.* 170:1869.

29. Zhou, T., H. Bluethmann, J. Eldridge, M. Brockhaus, K. Berry, and J.D. Mounz. 1991. Abnormal thymocyte development and production of autoreactive T cells in T cell receptor transgenic autoimmune mice. *J. Immunol.* 147:466.

30. Owen-Schaub, L.B., S. Yonehara, W.L. Crump, III, and E.A. Grimm. 1992. DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by *Fas* antigen engagement. *Cell. Immunol.* 140:197.

31. Miyawaki, T., T. Uehara, R. Nibu, T. Tsuji, A. Yachie, S. Yonehara, and N. Taniguchi. 1992. Differential expression of apoptosis-related *Fas* antigen on lymphocyte subpopulations in human peripheral blood. *J. Immunol.* 149:3753.

32. Adachi, M., R. Watanabe-Fukunaga, and S. Nagata. 1993. Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of *lpr* mice. *Proc. Natl. Acad. Sci. USA.* 90:1756.