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Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of *lpr* mice

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ABSTRACT **The mouse *lpr* (lymphoproliferation) mutation carries a rearrangement in the chromosomal gene for the Fas antigen, which mediates apoptosis. Isolation and characterization of mouse Fas antigen chromosomal gene from wild-type and *lpr* mice indicated an insertion of an early transposable element (ETn) in intron 2 of the Fas antigen gene of *lpr* mice. Hybrid transcripts carrying the Fas antigen and ETn sequences were expressed in the thymus and liver of the mutant. This indicated that premature termination and aberrant splicing of the Fas antigen transcript caused by the insertion of the ETn in the intron are responsible for the lymphoproliferation and autoimmune phenotype of the mutant mouse. On the other hand, an insertion of the ETn into an intron of a mammalian expression vector dramatically but not completely reduced the expression efficiency. These findings suggest that *lpr* mice are able to express a very low level of the Fas antigen.**

T-cell development occurs in the thymus where T cells reacting with autoantigen or T cells expressing nonfunctional T-cell receptors are deleted by apoptosis (1). Mice carrying the lymphoproliferation mutation (*lpr*) seem to have defects in the development of T cells (2). The phenotype in *lpr* mice includes production of multiple autoantibodies and accumulation of large numbers of nonmalignant $CD4^-$ $CD8^-$ T lymphocytes in lymph nodes and the spleen (2).

The Fas antigen is a cell surface protein that mediates apoptosis (3, 4). The Fas antigen consists of 319 amino acids (human) or 306 amino acids (mouse) and has similarity to the tumor necrosis factor receptors and the low-affinity nerve growth factor receptor (4, 5). These characteristics suggested that the Fas antigen is a receptor for an unknown ligand. Recently, Oehm *et al.* (6) isolated a cDNA for human Apo-1 antigen and showed that the Apo-1 antigen is identical to the Fas antigen. The murine Fas antigen gene was mapped near the *lpr* locus of mouse chromosome 19 (5). Little Fas antigen mRNA was detected in the thymus and liver of mice homozygous at the *lpr* locus, and a small but distinct rearrangement of the Fas antigen gene was observed in *lpr* mice (7). Furthermore, another allelic mutation of the *lpr* locus, lpr^{cg} (8), carried a point mutation in the cytoplasmic region of the Fas antigen gene, which abolished the ability of the Fas antigen to transduce the apoptotic signal (7).

In this report, we describe the isolation of the Fas antigen chromosomal genes from wild-type and *lpr* mice. Comparison of their structures indicated the insertion of an early transposable element (ETn) into intron 2 of the Fas antigen gene in *lpr* mice. This insertion caused premature termination of the Fas antigen mRNA at the long terminal repeat (LTR) region of the ETn and aberrant splicing of the Fas antigen mRNA. The ability of the ETn to suppress the gene expression was confirmed by inserting the ETn into an intron of a mammalian expression vector for granulocyte colony-stimulating factor (G-CSF) and expressing it in COS cells.

MATERIALS AND METHODS

Isolation of the Fas Antigen Chromosomal Genes. Genomic DNA prepared from the spleen of MRL +/+ or MRL *lpr/lpr* mice was partially digested with *Sau*3AI and fractionated on a 0.4% agarose gel. DNA from 10 kb to 20 kb was recovered and ligated with *Bam*HI-digested λEMBL3 arms. Recombinant DNA was packaged *in vitro* and λ phages were plated on *Escherichia coli* NM539. The libraries were screened using a ^{32}P-labeled DNA fragment carrying the sequence from positions 232 to 450 of mouse Fas antigen cDNA (pMF1) (5). The probe DNA was prepared by PCR using two oligonucleotides (AGGTACTAATAGCATCTCCG and CACAGTGTTCACAGCCAGGA) as primers and mouse Fas antigen cDNA as a template. After digestion of the product with *Mva* I, the 0.2-kb DNA fragment was labeled with ^{32}P by the random-primer labeling method. Plaque hybridization and preparation of recombinant phage DNAs were carried out by the standard procedures (9).

Southern Blot Hybridization, Northern Blot Hybridization, and Nucleotide Sequence Analysis. Chromosomal DNA prepared from wild-type and *lpr* mice was digested with appropriate restriction enzymes, electrophoresed on a 0.7% agarose gel, and transferred to a nitrocellulose filter (Schleicher & Schuell). Hybridization was carried out under stringency conditions as described (5). The 0.7-kb *Eco*RV–*Bam*HI fragment (Fig. 1*A*) was labeled with ^{32}P by the random-primer labeling method and used as a DNA probe.

Total cellular RNAs were prepared according to Chomczynsky and Sacchi (10) from the mouse liver or thymus. Northern blot hybridization was carried out as described (7). The probe DNAs were prepared by PCR using appropriate primers and pMF1 cDNA (5) as a template.

DNA sequencing was performed by the dideoxynucleotide chain-termination method using T7 DNA polymerase (Pharmacia) and deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (Amersham).

Analysis of RNA by PCR. The reverse PCR was carried out essentially as described (11). In brief, 2 μg of total RNA was used as a template for cDNA synthesis in 50 μl of reaction mixture with 0.5 μg of random hexamer and 80 units of avian myeloblastosis virus reverse transcriptase. After incubation at 42°C for 60 min, 5 μl of the reaction mixture was diluted with 100 μl of PCR buffer (12) containing 50 pmol of the forward primer and 50 pmol of the reverse primer. The PCR was performed using a DNA thermal cycler (Perkin–Elmer/Cetus) with 2.5 units of *Thermus aquaticus* DNA polymerase (*Taq* polymerase). The conditions for the PCR were 1.5 min

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Abbreviations: LTR, long terminal repeat; ETn, early transposable element; G-CSF, granulocyte colony-stimulating factor.

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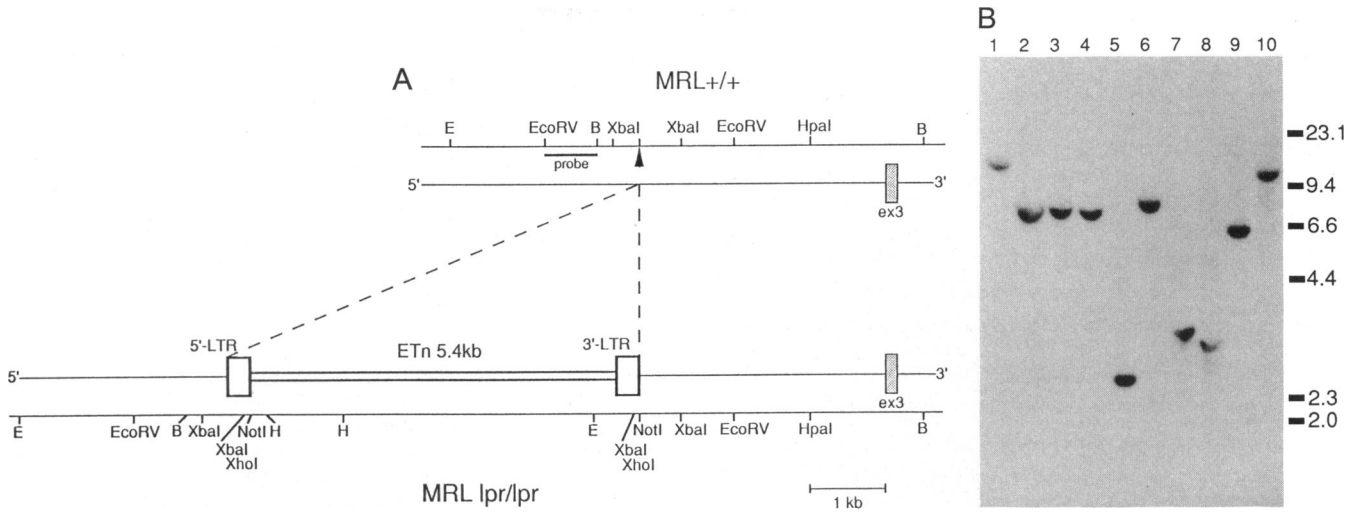

FIG. 1. Insertion of an ETn in intron 2 of the Fas antigen gene. (*A*) Restriction map of intron 2 in the Fas antigen gene from wild-type and *lpr* mice. The solid arrowhead indicates the inserted point. The shaded box represents exon 3 and the open boxes show the ETn LTRs. The probe DNA used for Southern blot hybridization is indicated by a bar. The recognition sites for major restriction enzymes are indicated. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII. (*B*) Southern blot hybridization analysis showing rearrangement of the Fas antigen gene in *lpr* mice. Genomic DNAs (10 μg per lane) from the wild-type (lanes 1, 3, 5, 7, and 9) or *lpr* mice (lanes 2, 4, 6, 8, and 10) were digested with *Eco*RI (lanes 1 and 2), *Bam*HI (lanes 3 and 4), *Eco*RV (lanes 5 and 6), *Bgl* II (lanes 7 and 8), or *Hpa* I (lanes 9 and 10). After separation on a 0.7% agarose gel, hybridization was carried out with the ^{32}P-labeled 0.7-kb *Eco*RV–*Bam*HI fragment (*A*).

at 95°C, 1.5 min at 70°C, and 2.0 min at 72°C for 30 cycles. The oligonucleotide primers were synthesized by a DNA synthesizer (Applied Biosystems model 381A).

Construction of Expression Plasmids, Transfection, and G-CSF Assay. The coding region of human G-CSF cDNA, 720 bp of the *Eco*RI–*Aha* III fragment of pBRV2 (13), was inserted at *Bst*XI site of pEF-BOS by using *Bst*XI adapters (designated pEF-BOS-GCSF). The 7.5-kb *Bam*HI–*Eco*RV fragment carrying part of intron 2 from the Fas antigen gene and the entire ETn (Fig. 1) was then inserted at the unique *Bgl* II site of intron 1 of the human elongation factor 1α gene (14) in pEF-BOS-GCSF in the right orientation (designated pBGMα) or the reverse orientation (designated pBGMβ).

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum. Approximately 1.2×10^5 cells were transfected with 10 μg of expression vector by the DEAE-dextran method as described (15). Seventy-two hours after transfection, the G-CSF activity in the medium was assayed by [^{3}H]thymidine incorporation into interleukin 3-dependent mouse myeloid NFS60 cells, as described (15).

RESULTS

Insertion of ETn in Intron 2 of the Fas Antigen Gene. We have previously isolated mouse chromosomal gene for Fas antigen from wild-type mice (R.W.-F. and S.N., unpublished results). This gene consists of >70 kb and has eight introns. Rearrangement of the Fas antigen gene in *lpr* mice was detected by Southern blot hybridization with a DNA probe containing nt 115–580 of mouse Fas antigen cDNA (7). To characterize the rearrangement of the Fas antigen gene in *lpr* mice, chromosomal gene libraries were constructed with spleen DNA from MRL +/+ and MRL *lpr/lpr* mice. These libraries were screened with the mouse Fas antigen cDNA (nt 232–450), and the positive recombinant DNAs were characterized by restriction enzyme mapping and Southern blot hybridization.

Comparison of the gene structures has indicated that an ≈5.4-kb DNA fragment was inserted 3.5 kb upstream of exon 3 in the Fas antigen gene in *lpr* mice (Fig. 1*A*). Southern blot hybridization analysis of genomic DNAs with the 0.7-kb *Eco*RV–*Bam*HI DNA fragment (Fig. 1*A*) showed that the 2.4-kb *Eco*RV and 5.6-kb *Hpa* I fragments in the wild-type DNA were replaced by 7.8-kb and 11-kb fragments in the *lpr* DNA, respectively (Fig. 1*B*), which indicated the insertion of a 5.4-kb fragment in the *lpr* DNA. The 13-kb *Eco*RI fragment in the wild-type DNA was replaced by a 7.4-kb fragment in *lpr* mice, which agrees with the presence of a single *Eco*RI site in the inserted DNA (Fig. 1*A*). The *Bam*HI-digested genomic DNAs gave 7.6-kb identical bands in wild-type and *lpr* mice, thus suggesting that no rearrangement had occurred in the 7.6-kb region upstream from the rearranged position.

Nucleotide sequences of the inserted DNA and its flanking regions were determined for the wild-type and *lpr* DNAs. The first 317 nt at the 5′ and 3′ ends of the inserted DNA had completely identical sequences that were homologous to the LTR of the mouse ETn (16, 17) (Fig. 2). The LTR carries an "AATAAA" polyadenylylation signal in the U3 region. A comparison of the nucleotide sequence of the ETn in the Fas antigen gene with the members of the ETn family indicated that the ETn in the Fas antigen gene was highly homologous to the RMg2 ETn (17). At the 5′ and 3′ ends of the inserted DNA, there was a 6-bp repeat sequence, CATGTG, that could be found once in the wild-type DNA (Fig. 2). From these results, it can be concluded that an RMg2-like ETn has been inserted in intron 2 of the Fas antigen gene of *lpr* mice by retrotransposition.

Aberrant Fas Antigen Transcripts in *lpr* Mice. Previously, we could detect (7) little Fas antigen mRNA in *lpr* mice by using the entire Fas antigen cDNA as a probe. However, since the promoter and exons 1 and 2 of the Fas antigen gene seem to be intact in *lpr* mice, the expression of Fas antigen gene was reexamined using a probe DNA containing that part of the Fas antigen cDNA. As shown in Fig. 3*A*, Northern blot hybridization using a probe DNA carrying exons 1 and 2 detected RNAs of ≈7.0, 4.0, and 1.0 kb, but little RNA of normal size, in the liver and thymus of *lpr* mice. When a probe DNA containing exons 3 and 4 was used, a 2.1-kb RNA was detected in wild-type mice whereas little hybridizing RNA was detected in *lpr* mice. These findings suggest that the Fas antigen mRNA is initiated normally in *lpr* mice, but then it is terminated before exon 3.

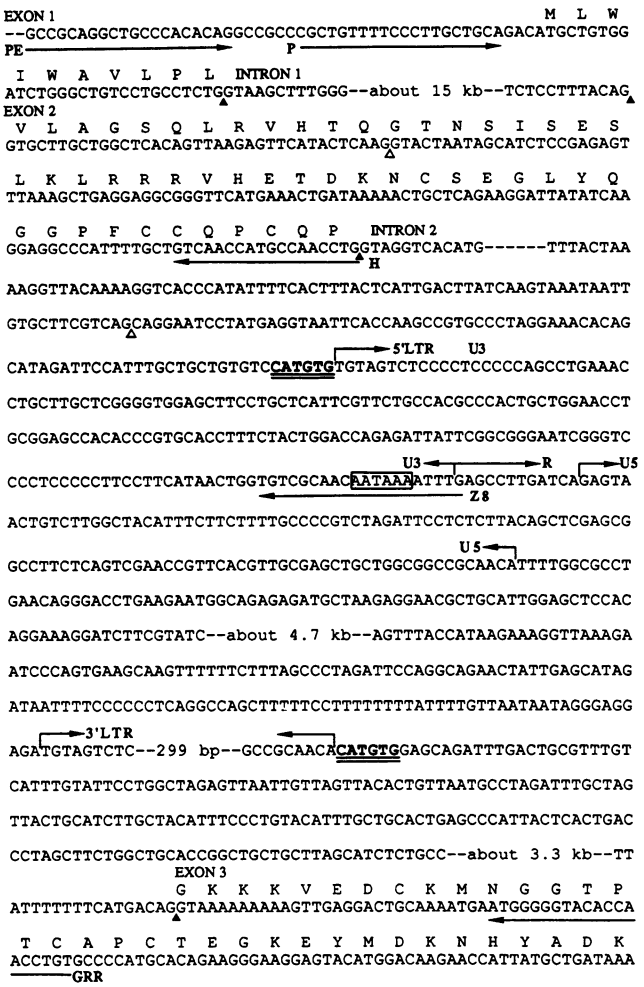

FIG. 2. Nucleotide sequences of exons 1–3 and the ETn in intron 2 of the Fas antigen gene in *lpr* mice. The junctions between exon and intron are indicated by solid triangles and the putative splice junctions for aberrant transcripts are indicated by open triangles. The AATAAA polyadenylylation signal in the LTR is boxed. The sequence of the 3′ LTR in ETn is identical to that of the 5′ LTR. The sequence of exons and intron 2 of the wild-type Fas antigen gene is identical to the corresponding sequence in *lpr* mice except for the ETn. The 6-bp repeats of the target sequence are doubly underlined. The oligonucleotide primers used for PCR in Fig. 3*B* are shown by arrows.

RNAs from the thymus of *lpr* mice were then analyzed by a reverse PCR using the primers indicated in Fig. 2. As shown in Fig. 3*B*, RNAs from the wild-type and *lpr* mice produced DNA fragments of 209 bp or 235 bp with combinations of primers in exon 1 (P or PE primer) and exon 2 (H primer). When the primer (Z8) complementary to the LTR of ETn was used as a reverse primer, RNA from the *lpr* mice but not from the wild-type mice gave DNA fragments of 350–680 bp or 380–710 bp using P or PE forward primers, respectively. On the other hand, when the primer complementary to the sequence of exon 3 in the Fas antigen gene (GRR primer) was used as a reverse primer, a 283-bp DNA fragment was detected with the wild-type RNA but not with the *lpr* RNA.

The PCR products amplified from the *lpr* RNA by using the primers in exon 1 and the LTR of ETn were subcloned into pBluescript vector and subjected to nucleotide sequence analysis. Among the 24 clones examined, three splice variants were found. As schematically described in Fig. 3*C*, all clones contained the 79-bp sequence localized upstream of the 5′ LTR and a part of the LTR. Most of the clones (22 of 24 clones) contained intact exons 1 and 2 of the Fas antigen gene (represented by clone F1-7). One clone (clone F2-9) contained an extra 158 nt of an unidentified sequence between exon 2 and the 5′ LTR, whereas the other clone (clone F2-10) had a truncated exon 2. The sequences at the junction of the putative exons were in agreement with the consensus donor or acceptor splice sequences (18) (Fig. 2), suggesting these RNAs had originated by aberrant splicing. These findings indicate that the transcripts initiated at exon 1 of the Fas antigen gene in *lpr* mice are terminated at the LTR region [probably at the poly(A) adenylylation site] of the ETn inserted in intron 2 of the gene. The premature RNA is then spliced aberrantly to produce a short mRNA of ≈1 kb. The larger RNA species (4 kb and 7 kb) detected by Northern blot hybridization in *lpr* mice (Fig. 3*A*) may be unspliced RNAs or RNAs that escape termination at the 5′ LTR.

Effect of an ETn Insertion on Gene Expression. We then examined the effect of an insertion of the ETn on expression of other genes. Plasmid pEF-BOS-GCSF is a human G-CSF (13) expression plasmid constructed with the pEF-BOS vector (19). The pEF-BOS expression vector carries exon 1, intron 1, and exon 2 from the human elongation factor 1α gene (14) (Fig. 4*A*). The 7.5-kb *Bam*HI–*Eco*RV fragment carrying the entire ETn sequence in intron 2 of the Fas antigen gene (Fig. 1*A*) was inserted at the unique *Bgl* II site in the intron of pEF-BOS-GCSF in the right orientation (pBGMα) or reverse orientation (pBGMβ) (Fig. 4*A*). The resultant plasmid DNAs were introduced into COS-7 cells, and the G-CSF activities secreted into the medium were assayed. As shown in Fig. 4*B*, the pEF-BOS-GCSF expression vector produced G-CSF at ≈14,000 units/ml. The insertion of the ETn in a reverse orientation (pBGMβ) had almost no effect (at most 40% reduction) on the production of G-CSF. On the other hand, the plasmid pBGMα produced G-CSF at only 250 units/ml, which is ≈60 times less than that produced with pEF-BOS-GCSF. These findings indicate that the ETn has the ability to reduce gene expression if it is inserted in the intron in the right orientation. To confirm this result, pEF-BOS-GCSF, pBGMα, or pBGMβ was introduced into mouse L929 cells, and stable transformants carrying these plasmids were constructed. The L929 cell transformant carrying pEF-BOS-GCSF or pBGMβ produced G-CSF constitutively at an efficiency of 35 units per ml per day, whereas the G-CSF production rate by the L929 cell transformants carrying pBGMα was only 1.0 unit per ml per day.

DISCUSSION

In this report, we have shown that the Fas antigen gene in *lpr* mice carries the insertion of an early transposable element in intron 2. This insertion causes premature termination and aberrant splicing of the Fas antigen mRNA in *lpr* mice. Actually, insertion of the ETn into an intron of a mammalian expression vector dramatically reduced the expression efficiency. However, it did not completely abolish gene expression, suggesting that a small amount of the transcript can be read through the ETn region and spliced correctly. This result suggests that *lpr* mice have the ability to express a very low level of the Fas antigen, although we could not detect intact Fas antigen mRNA in this mouse by Northern blot hybridization and PCR.

The Fas antigen is expressed in the thymus and mediates apoptosis. Since the mutation in the Fas antigen gene (*lpr* mutation) causes abnormal accumulation of $CD4^-$ $CD8^-$ T-lymphocytes and autoimmune disease, we have suggested that the Fas antigen is involved in clonal deletion of T cells in the thymus (7). However, it is controversial whether mice carrying the *lpr* mutation have defects in the clonal deletion of T cells. Matsumoto *et al.* (20) and Zhou *et al.* (21) have observed some intrinsic defects in clonal deletion in T cells derived from *lpr* mice. On the other hand, Kotzin *et al.* (22),

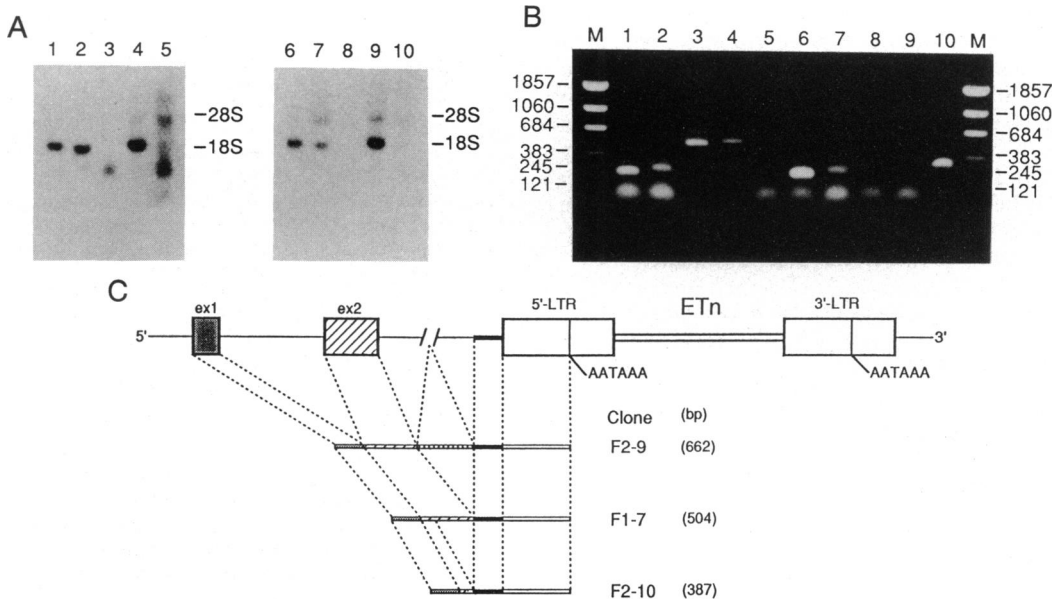

FIG. 3. Aberrant transcripts of the Fas antigen gene in *lpr* mice. (*A*) Northern blot hybridization analysis of RNAs in *lpr* and wild-type mice by using the probe DNA containing exons 1 and 2 (lanes 1–5) or exons 3 and 4 (lanes 6–10). Total cellular RNAs (9 μg) are from the liver (lanes 2, 3, 7, and 8) and thymus (lanes 4, 5, 9, and 10) of MRL/MpJ +/+ (lanes 2, 4, 7, and 9) and MRL/MpJ *lpr/lpr* (lanes 3, 5, 8, and 10). Total cellular RNA (4.5 μg) from mouse L929 cells was also analyzed (lanes 1 and 6). The probe DNA for exons 1 and 2 or 3 and 4 carries the sequence of nt 1–233 or nt 232–450 of the mouse Fas antigen cDNA (5), respectively. (*B*) Aberrant transcripts in *lpr* mice detected by reverse PCR. Single-stranded cDNA was synthesized using total cellular RNAs from the thymus of *lpr* mice (lanes 1–5) or wild-type mice (lanes 6–10), and the cDNA containing the Fas antigen sequence was amplified with various combinations of primers. The primers used are as follows: Lanes, 1 and 6, primers P (CGCTGTTTTCCCTTGCTGCA) and H (CAGGTTGGCATGGTTGAC); 2 and 7, primers PE (GCCGCAGGCTGCCCA-CACG) and H; 3 and 8, primers P and Z8 (CAAATTTTATTGTTGCGACA); 4 and 9, primers PE and Z8; 5 and 10, primers PE and GRR (ACAGGTTGGTGTACCCCCAT). The products were analyzed on a 1.2% agarose gel and visualized by ethidium bromide fluorescence. Size markers (base pairs) are shown. (*C*) Structure of aberrant Fas antigen transcripts in *lpr* mice. The chromosomal gene structure of the Fas antigen gene in *lpr* mice (at the top) and the structures of three cDNAs amplified by reverse PCRs of the thymus RNA in *lpr* mice are schematically shown. The lengths of the amplified cDNA clones are indicated in parentheses in base pairs.

Singer *et al.* (23), and Sidman *et al.* (24) have reported that the *lpr* mice have the potential to delete autoreactive T cells. These conflicting observations may be partly explained by leakiness and fluctuations of the Fas antigen expression in *lpr* mice. Furthermore, slightly different autoimmune phenotypes in different mouse strains (25) may also be due to the various efficiencies of premature termination of Fas antigen mRNA observed in the ETn region.

The ETns are a family of murine retrotransposon-like elements, and ≈1000 copies are present in the mouse genome (26). These elements are abundantly transcribed in early mouse embryogenesis although they contain no significant open reading frames to code for proteins involved in their transposition (16). Nevertheless, ETn insertion was found in various mouse chromosomal loci such as the T gene (*Brachyury*) (27) and the major mammalian skeletal muscle

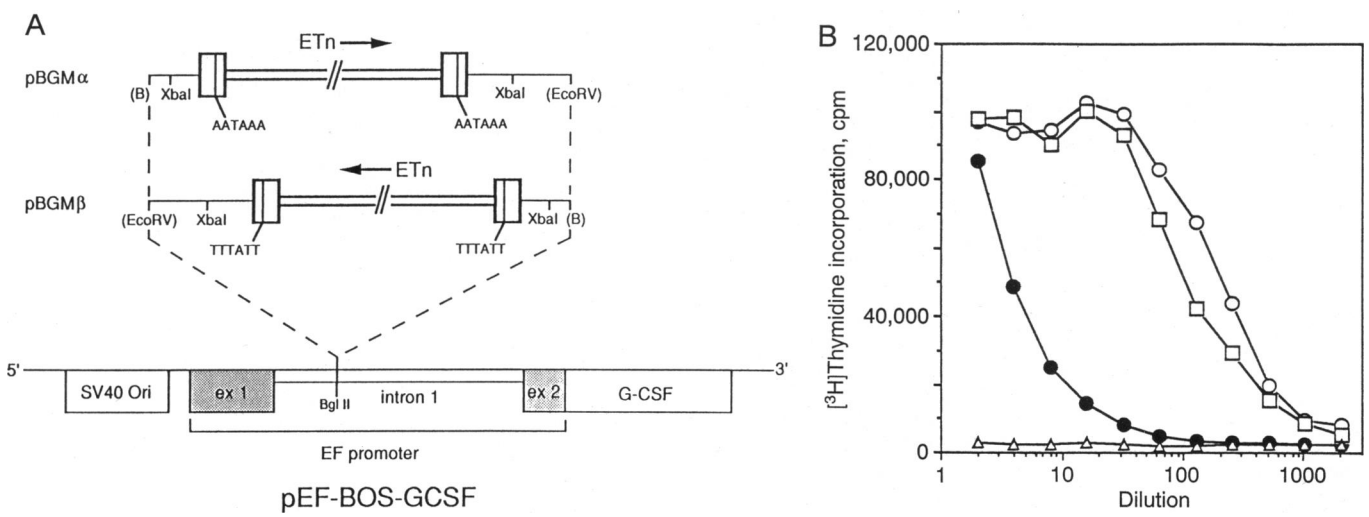

FIG. 4. Reduction of gene expression by insertion of ETn into an intron. (*A*) Construction of the expression vector carrying ETn. The G-CSF expression vector (pEF-BOS-GCSF) and its derivatives carrying the ETn in the right orientation (pBGMα) or in the reverse orientation (pBGMβ) are schematically shown. (*B*) The G-CSF activity accumulated in the medium of COS-7 cells transfected by expression vectors. COS-7 cells were transfected with 10 μg of the expression plasmids of pEF-BOS (△), pEF-BOS-GCSF (○), pBGMα (●), or pBGMβ (□). Seventy-two hours after transfection, an aliquot of the medium was diluted 1:50, and the G-CSF activity was assayed after serial dilution of 1:2.

chloride channel gene (28). These insertions also cause the respective phenotype of loss of function in mice. A similar mechanism for the Fas antigen gene in *lpr* mice seems to apply to these mutants. These findings further suggest that the ETn can be used as a mutagen to produce loss-of-function mutant mice by using methods similar to those described by Gossler *et al.* (29), Friedrich and Soriano (30), and Skarnes *et al.* (31). It should be possible to introduce the ETn into mouse embryonal stem cells and produce mouse homozygotes carrying the ETn at various loci in the mouse genome.

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