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Isolation of a cDNA clone encoding a novel membrane protein expressed in lymphocytes

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Abstract By subtractive hybridization using single-stranded phagemids with directional inserts, we isolated a mouse cDNA clone, LSM-1, from temperature-sensitive Abelson virus-tranformed immature B cells whose differentiation was being induced after the shift from the permissive (35°C) to the non-permissive temperature (39°C). LSM-1, which encodes an as yet unknown peptide of 197 amino acids, has a putative signal sequence and a trans-membrane region, and is expressed in B- and T-cell lines, in spleen, thymus, and bone marrow of adult mice, and in embryos.

Key words: Lymphocyte-specific cDNA; Embryonic stage-specific cDNA; Subtractive hybridization; Single stranded phagemid

1. Introduction

Lymphocytes originate from multipotential hematopoietic stem cells through various stages of differentiation. This differentiation is thought to progress through serial stage-specific expression of various genes. Therefore, isolation of the genes which are expressed stage-specifically is essential for the elucidation of the mechanisms of the differentiation from multipotential hematopoietic stem cells to lymphocytes.

In previous studies, we have established SPL2-1-2 [1–6], an immature B cell line transformed with a temperature-sensitive mutant of Abelson murine leukemia virus [7,8]. This cell line starts to differentiate to more differentiated cells after the shift of culture temperature from the permissive (35°C) to the non-permissive temperature (39°C). During this differentiation process, a variety of genes will be specifically expressed.

In this study, we attempt to isolate the genes that drive the differentiation of immature B cells by using subtractive hybridization between terminally differentiated myeloma cells and SPL2-1-2 immature B cells whose differentiation was being induced by the shift of culture temperature and describe the isolation of a cDNA clone expressed in lymphocytes and at the embryonic stage.

2. Materials and methods

2.1. Cells

SPL2-1-2-12 used here for the construction of cDNA library was a subclone of SPL2-1-2 [1–6], which was an immunoglobulin-negative immature B cell line transformed with a temperature-sensitive mutant of Abelson murine leukemia virus [7,8]. A pre-B cell line (70Z/3 [9]), mature B cell lines (BCL_1 [10] and WEHI-231 [11]), plasma cell lines (P3U1 [12] and MPC-11 [13]), T cell lines (Lyb-1 [14] and EL-4 [15]), monocyte cell line ($p388D_1$ [16]), myeloid cell line (WEHI-3 [17]), and Friend erythroleukemia cell line (TSA-8[18]) were cultured in RPMI 1640 medium containing 5% fetal bovine serum, 2 mM glutamine, and 5×10^{-5} M 2-mercaptoethanol. Fibroblast cell lines (NIH3T3, and ANN-1 [19]) were grown in Eagle's MEM containing 5% fetal bovine serum and 2 mM glutamine.

2.2. cDNA cloning vector system: construction and screening of a cDNA library

The original Okayama-Berg plasmid vectors [20], pcDV1 and pL1 were modified to generate vector-primer and linker DNAs as follows (Giri, C.P., Ogawa, H., Sasser, V., Spillare, B. and Harris, C.C., unpublished data). To allow for in vitro synthesis of sense and antisense RNA transcripts from the cDNA inserts and to perform subtraction in sufficient enough quantities, SP6 and T7 bacteriophage RNA polymerase promoter sequences were inserted in opposite transcriptional orientation into the vector-primer (pcDV1) and linker plasmid (pL1), and the modified vector-primer and linker plasmid were designated as pLHC2-HO2 and pLHC2-CG5, respectively.

pLHC2-HO2 with the SP6 promoter sequence was constructed from pcDV1, and then a dT-tailed pLHC2-HO2 vector-primer was prepared by the addition of an average of 45 dT residues which were covalently linked to the 3'-ends after linearization by SacI digestion of pLHC2-HO2.

The 521-bp *Pst*I/*Hin*dIII linker DNA fragment was excised from the plasmid pL1, gel purified, and then recloned into *Pst*I/*Hin*d III-digested pGEM4 cloning vector to use certain restriction sites of this vector in the further experiments (designated as pLHC2-CG4). pLHC2-CG5 was constructed from pLHC2-CG4 by the insertion of T7 promoter sequence into the polylinker site, and then pLHC2-CG6 was prepared from pLHC2-CG5 by the insertion of the f1 origin. Moreover, an average of 9 dC residues were tailed to the 3'-end of the linearized pLHC2-CG6 using terminal deoxy-transferase. After *Hin*dIII digestion, a 586-bp dC-tailed DNA fragment that contained both the f1 origin and T7 promoter sequence was purified and used as a linker.

Using 2 μg of the vector primer and 1 μg of the linker, SPL2-1-2-12 cDNA library (1.0×10^6 independent clones) was constructed as previously described [20] from poly(A)$^+$ RNA (5 μg) of SPL2-1-2-12 cells that were continued to culture for 7 days at the non-permissive temperature (39°C) to induce cell differentiation.

Next, an enriched cDNA library was constructed as previously described [21]. Briefly, single-stranded circular DNAs containing antisense cDNA sequences were prepared from the SPL2-1-2-12 cDNA library by infection of R408 strain of helper phage M13, and then hybridized to biotinylated poly(A)$^+$ RNA from a myeloma cell line, P3U1. After hybridization, streptavidin was added, and streptavidin–biotin–RNA and streptavidin–biotin–RNA–DNA complexes were removed by extraction with phenol/chloroform [22]. The single-stranded DNA was recovered from the aqueous phase by ethanol-precipitation, and converted to double-stranded plasmid DNA containing cDNA inserts by a DNA polymerase reaction primed with the T7 promoter sequence. Finally, the double-stranded plasmid DNA was transfected

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into *E. coli*, and a cDNA library, which we have termed 'enriched cDNA library', was prepared.

To isolate cDNA clones that are expressed specifically in SPL2-1-2-12, but not in P3U1, the enriched cDNA library was screened by using a ^{32}P-labeled subtracted single-stranded cDNA probe, which was prepared as follows. A single-stranded cDNA was synthesized from 5 μg of poly(A)$^+$ RNA of SPL2-1-2-12 by using oligo-dT primer and AMV reverse transcriptase, and hybridized to 50 μg of biotinylated poly(A)$^+$ RNA of P3U1. After hybridization followed by the addition of streptavidin, the single stranded cDNA was recovered as described above and used as the DNA probe.

Approximately 1×10^3 transformants from the enriched cDNA library were grown, and replicate nylon filters blotted with the colonies were prepared, lysed with alkali, hybridized with the ^{32}P-labeled subtracted single-stranded cDNA probe, and autoradiographed. The colonies that were positive on the replicate nylon filters were picked up, and the cDNA inserts were recovered and sequenced.

2.3. Northern blot analysis

Total RNA (10–20 μg) was used for Northern blot analysis on 1.2% agarose gels containing 6.7% formaldehyde. Gels were washed twice with 10 × SSC for 20 min, and RNA was transferred to nylon membrane filters in 20 × SSC. Hybridization was performed using the isolated cDNA labeled with ^{32}P as a DNA probe in 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 1% SDS, and 200 μg/ml denatured salmon sperm DNA at 42°C. The filters were washed sequentially in 2 × SSC and 1% SDS at room temperature for 40 min, in 0.2 × SSC and 1% SDS at room temperature for 40 min, and then in 0.2 × SSC and 1% SDS at 65°C for 30 min. Autoradiography was performed for 2–7 days at −70°C.

2.4. DNA sequencing

cDNA inserts were sequenced by dideoxy chain termination method. The sequences presented here were determined by bidirectional sequencing.

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          10          20          30          40          50          60
CGGCACGAGCCGTGCAAAAGCCATCGGATCAATGGCTCTGCCTGGTACCCTCAGATTTGG
                                  M   A   L   P   G   T   L   R   F   G

          70          80          90         100         110         120
GGTGCTGATGGCCCTGCCAGGGGCACTGGCCTCTGGGGCAGACCCAGAGGACGGTGTGGG
 V   L   M   A   L   P   G   A   L   A   S   G   A   D   P   E   D   G   V   G

         130         140         150         160         170         180
CTCCAGTGTGGTCACCATTGTCCTGCTGCTCTTGCTCCTGCTGCTGCTGGTCACTGCCCT
 S   S  [V   V   T   I   V   L   L   L   L   L   L   L   L   V   T   A   L

         190         200         210         220         230         240
AGCCTTGGCTTGGCGTCGCCTCAGCCATGCCTCAGGGGGGTACTACCACCCAGCTCGCCT
 A   L   A   W] R   R   L   Ⓢ   H   A   S   G   G   Y   Y   H   P   A   R   L

         250         260         270         280         290         300
GGGTGCTGCCTTGTGGGGCCACACCTGCCGCCTGCTCTGGGCCAGCCCTGCAGGCCGCTG
 G   A   A   L   W   G   H   T   C   R   L   L   W   A   S   P   A   G   R   W

         310         320         330         340         350         360
GCTTCGCGCCCGCACTGAGCTGGAATCCCCAGAAGAATCAGGGCCGCCGGAGGATGAAGA
 L   R   A   R   T   E   L   E   S   P   E   E   S   G   P   P   E   D   E   E

         370         380         390         400         410         420
GGATGCAGAAGATTTTGTGATAGATGGTGGCCCCGAGGAAGCTGCCGCCAAGGAAGAGGA
 D   A   E   D   F   V   I   D   G   G   P   E   E   A   A   A   K   E   E   E

         430         440         450         460         470         480
GCAGCGGTGTCAAGCAGAGCAGACCCGAGATCCACGTGACACAGACAGTGACGGGGGCCT
 Q   R   C   Q   A   E   Q   T   R   D   P   R   D   T   D   S   D   G   G   L

         490         500         510         520         530         540
GGGCCTGAGCTCTCAGGGTCCTGTGGGCTCAGGCAGCAGCGCCGAGGCCCTGCTGAGTGA
 G   L   S   S   Q   G   P   V   G   S   G   S   S   A   E   A   L   L   S   D

         550         560         570         580         590         600
CCTGCATGCCTTTTCTGGTAGTGCTGCCTGGGATGACAGCGCTGGAGGAGCAGGGGGCCA
 L   H   A   F   S   G   S   A   A   W   D   D   S   A   G   G   A   G   G   Q

         610         620         630         640         650         660
GGGCCTCCGGGTCACTGCACTATAGGAGTCAGCTTGTCCTGTTCCTGCCCCAGCCTCTGA
 G   L   R   V   T   A   L   ***
         670         680         690         700         710         720
CTTTTCCCATGCATGTTATGGTGAAACACAAGCTGCCCTGGACCTTCGTCACCCCACCCT

         730         740         750         760         770         780
TACATCAGCTTCTTAGACTGACACCCCAACCAGTTCCCCAGAGTGTGTGTGTGCATCCCA

         790         800         810         820         830         840
GACAGGCACTGGGCAAACATTAAAAATAAAACTTTGTAAGCACTGAAAAAAAAAAAAAAA
                             ******
         850
AAAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide sequence of LSM-1 cDNA. Deduced amino acid sequence is described in single-letter codes. A dashed line and a box represent a putative signal peptide and a transmembrane region, respectively. Oligonucleotide primer sequences used for primer extension analysis are underlined. Asterisks and a dotted line represent a termination codon and a polyadenylation signal, respectively. A possible phosphorylation site is circled.

2.5. Primer extension analysis

Synthetic oligonucleotides were labeled to a specific activity of 1.5–1.8 × 10^8 cpm/μg with T4 polynucleotide kinase and [γ-^{32}P]ATP. Ten ng of the labeled oligonucleotide were annealed to 4 μg of poly(A)$^+$ RNA of SPL2-1-2-12 in 40 mM PIPES (pH 6.5), 0.4 M NaCl, 1 mM EDTA, and 0.2% SDS. For annealing, the reaction mixture was heated at 85°C for 5 min and then incubated at 42°C overnight. Primer extension experiments were carried out by the addition of reverse transcriptase to the above reaction mixture, followed by the incubation at 42°C for 90 min, and the products were analyzed on 6% polyacrylamide gels under the denatured conditions.

2.6. In situ hybridization

In situ hybridization was performed as previously described [23]. To prepare cRNA probe (Ribo probe), LSM-1 cDNA was inserted into a SP64 plasmid vector in message-sense and -anti-sense orientations. Transcription was performed to a specific activity of 10^8–10^9 cpm/μg DNA template in 10 μl of reaction solution containing 1 μg of linearized DNA template, 25 μM [^{35}S]uridine triphosphate, 500 μM each of ATP, CTP and GTP, 20 units of human placental RNase inhibitor, and 20 units of SP6 polymerase, and then the probe was purified on a Sephadex G-100 column.

Sections were postfixed by exposure to formaldehyde vapours for 30 min, overlaid with approximately 100 μl of hybridization solution containing 50% formamide and 3 × 10^6 cpm/ml of denatured cRNA probe, and incubated at 50°C overnight. On the following day, the sections were washed, treated with 60 μg/ml RNase A at 37°C for 45 min, and then washed stringently in 2 × SSC at 50°C for 1 h, followed by 0.1 × SSC at 50°C for 3 h. After overnight washing at room temperature, the sections were dehydrated through graded ethanol containing 0.3 M ammonium acetate, vacuum dried, and exposed to X-ray film for 1–10 days. They then were dipped in NTB2 nuclear track emulsion and stored in the dark at 4°C for 2–4 weeks.

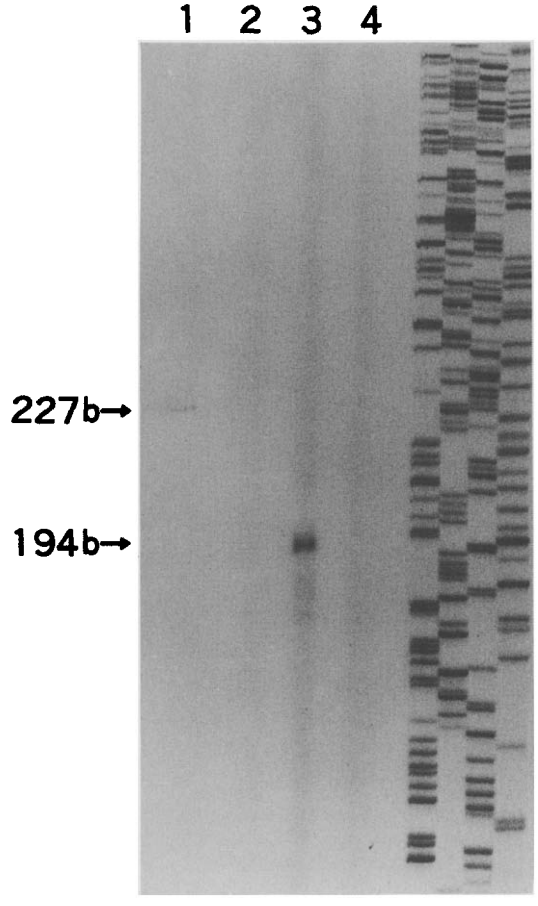

Fig. 2. Primer extension analysis of LSM-1 mRNA. Lanes 1 and 2: extension products from SPL2-1-2-12 mRNA and tRNA using 24-mer primer, respectively (see Fig. 1). Lanes 3 and 4: extension products from SPL2-1-2-12 mRNA and tRNA using 26-mer primer, respectively (see Fig. 1).

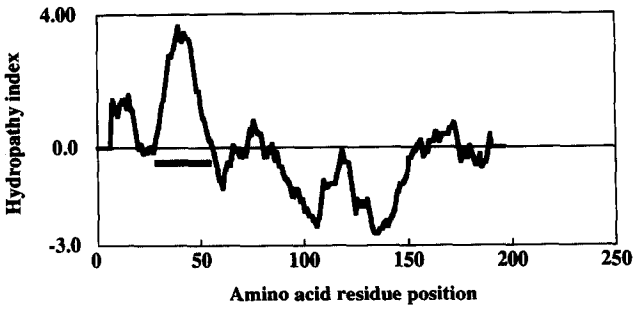

Fig. 3. A Hydropathy plot of the inferred amino acid sequence of LSM-1 product. The hydropathic profile was described by the algorism of Kyte and Doolittle with a window setting of 15 residues. A thick line represents a proposed transmembrane region.

3. Results and discussion

The enriched cDNA library was screened using the ^{32}P-labeled subtracted single-stranded cDNA probe. Out of 179 transformants positive for colony hybridization, 27 were randomly selected for further characterization. cDNA inserts were recovered from these 27 transformants and tested for its expression in SPL2-1-2-12, P3UI and ANN-1 using Northern blot analysis. Five out of the 27 cDNA clones hybridized only to SPL2-1-2-12 RNA, but not to P3U1 and ANN-1 RNA. Finally, we selected a cDNA clone with unknown DNA sequence for further characterization. Using this cDNA clone as a DNA probe, we screened λgt10 SPL2-1-2-12 cDNA library, which was prepared according to the manufacture's directions [24], to isolate the full-sized cDNA clone. Twenty out of 3.5 × 10^5 plaques were positive for the hybridization, and the longest cDNA insert, named LSM-1, was recovered from the phage clone. LSM-1 was subcloned into plasmid and sequenced. Sequencing analysis of LSM-1 showed that LSM-1 consisted of 859 base pairs and the longest open reading frame encoded 197 amino acids (Fig. 1). References to current data base revealed that LSM-1 had no significant sequence homologies to known genes.

To confirm that LSM-1 has an open reading frame from ATG at bp 32–34 to TAG at bp 623–625, primer extension analysis was performed by using partial sequences of LSM-1 as primers (Fig. 2). The primer ranging from bp 150 to 175 produced 194-base products while the primer ranging from bp 185 to 208 produced 227-base products, showing that the extension was terminated at the same position in two different primers. These results confirmed that LSM-1 had a single open reading frame beginning with ATG at bp 32–34 and showed that LSM-1 mRNA had a 50-nucleotide 5'-non-translated sequence.

A hydropathy profile of LSM-1 protein was described on the basis of the deduced amino acid sequences (Fig. 3). The peptide contains hydrophobic stretches capable of forming a signal and a transmembrane sequence. We assigned a potential cleavage site for the signal peptide between the alanine and the aspartate according to the −3, −1 rules of signal peptidase specificity [25–27]. The most hydrophobic region from position +33 to

+54 that flanked on two basic amino acids (Arg-Arg) on the carboxyl side was predicted as a transmembrane domain [28]. Since basic sequences are often found on the cytoplasmic side of membrane proteins, the basic sequence extending from the transmembrane domain to the carboxyl terminus would be a cytoplasmic domain. Serine at position 58 would be a possible recognition site for cAMP-dependent protein kinase [29]. These results suggest that the mature protein of LSM-1 product is a membrane protein of 174 amino acids with a predicted molecular weight of 18 kDa and having an extracellular and a cytoplasmic domain of 9 and 143 amino acid residues, respectively.

An example of a membrane protein with a small extracellular domains is the ζ (zeta) chain of T-cell receptor complex [30]. This protein contains 9 amino acids in the extracellular domain and 113 amino acids in the cytoplasmic domain and plays a role in efficient cell-surface expression and function of T-cell receptor complex. Therefore, we speculate that LSM-1 protein might interact with other membrane proteins and have some role in signal transduction.

To determine the lineage-specific expression of LSM-1, the expression of LSM-1 in various cell lines was examined by Northern blot analysis (Fig. 4A). Immature B (SPL2-1-2-12), pre-B (70Z/3), mature B (BCL1 and WEHI-231) and T (Lyb-1 and EL-4) cell lines expressed LSM-1. However, myeloma cell lines (P3U1 and MPC-11), monocyte cell line (P388Dl), myeloid cell line (WEHI-3), Friend erythroleukemia cell line (TSA-8) and fibroblast cell lines (NIH3T3 and ANN-1) did not express LSM-1. When the expression of LSM-1 in various tissues of Balb/c adult mice and in embryos was examined, LSM-1 was strongly expressed in spleen and thymus of adult mice and in the whole embryos of days 12 and 16, and weakly expressed in bone marrow, but not in brain, kidney, liver, and muscle (Fig. 4B,C). Hybridization with a β-actin probe confirmed equivalent RNA loading in each lane (data not shown).

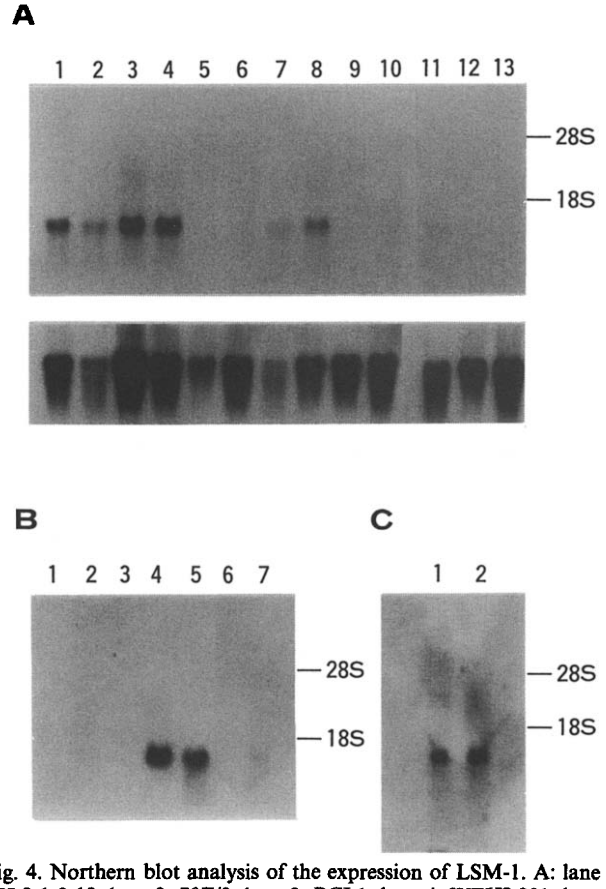

Fig. 4. Northern blot analysis of the expression of LSM-1. A: lane 1, SPL2-1-2-12; lane 2, 70Z/3; lane 3, BCL1; lane 4, WEHI-231; lane 5, P3U1; lane 6, MPC-11; lane 7, Lyb-1; lane 8, EL-4; lane 9, P388D1; lane 10, WEHI-3; lane 11, TSA-8; lane 12, NIH3T3; lane 13, ANN-1. RNAs were hybridized to the LSM-1 (upper) and β-actin (lower) DNA probes. B: lane 1, brain; lane 2, kidney; lane 3, liver; lane 4, spleen; lane 5, thymus; lane 6, muscle; lane 7, bone marrow. C: lane 1, day 12-embryo; lane 2, day 16-embryo.

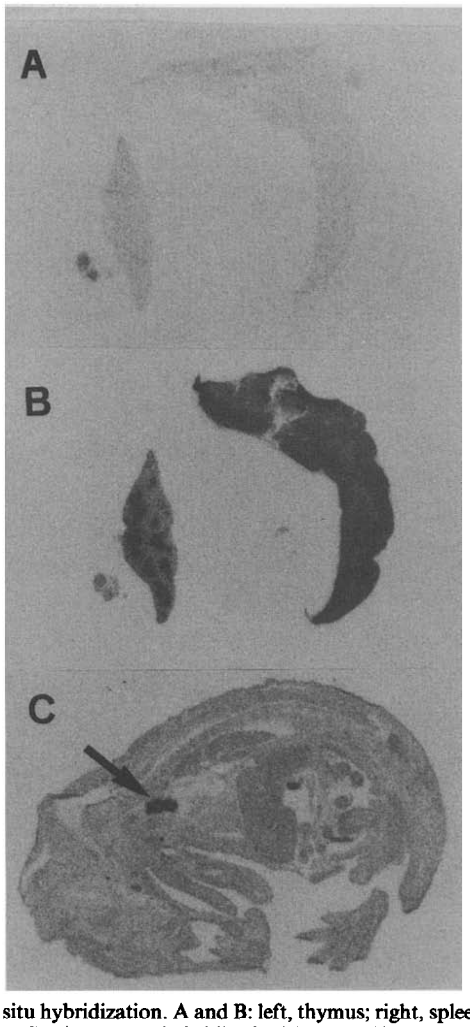

Fig. 5. In situ hybridization. A and B: left, thymus; right, spleen. C: day 15-embryo. Sections were hybridized with sense (A) or anti-sense (B and C) probes. An arrow indicates the thymus.

In situ hybridization was performed to analyse the localization of LSM-1 expression (Fig. 5). Spleen and thymus of adult mice diffusely expressed LSM-1 with many nodules of abundant expression. Also, day 15-embryo of mice diffusely expressed LSM-1. Especially, thymus of the embryo very strongly expressed LSM-1.

Taken together, these results showed that LSM-1 was expressed in both lymphocytes and embryos. LSM-1 does not have homology with any other proteins expressed in lymphocytes and its function is not known at present.

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