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LL55: Selective Early Lymphoid Expression of a Murine cDNA Clone Which Encodes a Putative RNA-Binding Protein

Takashi Baba, Saori Mitsuyoshi*, Hideya Igarashi* and Nobuo Sakaguchi*

*Department of Immunology and Department of Ophthalmology, Faculty of Medicine, Tottori University, Yonago 683 and *Department of Immunology, Kumamoto University School of Medicine, Kumamoto 860, Japan*

Specific expression of various RNA-binding protein (RNP) molecules is one of the critical factors that determine the development in *Drosophila melanogaster* and *Caenorhabditis elegans*. In the present study, we found a complementary DNA clone, LL55 encoding a putative RNP. Structural comparison of nucleotide and amino acid sequences demonstrate that LL55 encoding a nuclear protein with two RNA-binding consensus motifs and the common arginine-serine repeats, is similar to the sex-determining transformer-2 (tra-2) gene of *Drosophila melanogaster*. The expression of 2.3 kilobase (kb) and 1.5 kb LL55 messenger RNA (mRNA) is ubiquitous in murine organs and tissues, but it is selective in the early development of fetal life. It appears as early as day 10.5 and increases until day 15 of gestation in accordance with the expression of λ5 mRNA which is involved in murine pre-B cell generation. Taking account of the selective expression and the specific regulation of RNP molecules, the finding of LL55 RNP provides a new outlook for the generation and function of the immune system.

Key words: B cell; cDNA clone (LL55); fetal development; mRNA splicing; RNP

Precursors of murine lymphocytes are generated from multipotent haematopoietic stem cells in the fetal liver or in the adult bone marrow (Moore and Metcalf, 1970; Huang et al.,1994). The earliest identifiable precursor cells appear as early as day 10 in the area of the yolk sac (blood islet), and then rapidly expand into the fetal liver by day 13 (Moore and Metcalf, 1970; Strasser et al., 1986). These progenitor cells show surface characteristics like $CD34^{-}$ $Lin^{-}Sca1^{+}Thy1^{low}$ cells, with germ line configurations of antigen receptor genes (Smith et al., 1991). Such lymphoid progenitor cells undergo molecular events for gene rearrangements of immunoglobulin (Ig) or T cell receptor (TCR) loci, generating the precursor cells in the fetal liver or thymus by day 13 to day 16 (Ogawa et al., 1988; Penit and Vasseur, 1989; Rolink and Melchers, 1991). During the development of lymphoid lineage cells, a number of cell surface molecules appear as CD117 (c-kit), CD44 (Pgp-1) and CD25 (IL-2Rα) for T lineage cells (Pearse et al., 1989; Wu et al., 1991; Godfrey and Zlotnik, 1993; Hozumi et al., 1994) and as CD43 and CD45 (B220) for B lineage cells (Hardy et al., 1991; Osmond et al., 1994). Although several studies have defined molecules involved in the generation of lymphoid lineage cells by the gene targeting experiments (Kitamura and Rajewsky, 1992; Mombaerts et al., 1992; Shinkai et al., 1992; Bain et al., 1994; Georgopoulos et al., 1994; Scott et al., 1994; Zhuang et al., 1994), it is still not clear what the molecular mechanism that controls the early development of lymphoid lineage cells is.

Specific expression of various RNA-binding protein (RNP) molecules is one of the critical factors that determine the development in *Drosophila melanogaster* and *Caenorhabditis elegans* (Parkhurst and Meneely, 1994;

Abbreviations: bp, base pair; cDNA, complementary DNA; Ig, immunoglobulin; kb, kilobase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; mRNA, messenger RNA; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RAG-1, recombination activating gene-1; RNP, RNA-binding protein; TCR, T cell receptor; tra-2, transformer-2

Marx, 1995; Ryner and Swain, 1995).To study the stage specific regulation of cell development, we screened a complementary DNA (cDNA) library of a murine embryo (day 10.5) and identified a cDNA clone named LL55 encoding an RNP that is expressed selectively during early embryonal development. In the present study we determined the structure and the expression of LL55 and described the structural similarity with sex-determining RNP molecule in *Drosophila melanogaster* (Belote and Baker et al., 1983; Amrein et al., 1988, 1990).

Materials and Methods

Isolation of cDNA clones from fetal cDNA library

5×10^5 cDNA clones of a murine fetal (day 10.5) cDNA library constructed in λgt10 (obtained from CLONTECH Laboratories Inc., Palo Alto, CA) were screened by differential screening with radioactive cDNAs from mRNAs of an adult liver, thymus, spleen, bone marrow, WEHI-231 B cell line and BW5147 T cell line. Positive clones with only lymphoid probes were isolated. Nearly 800 clones were isolated and their individual inserts were tested by the polymerase chain reaction (PCR) method using λgt10 forward 27mer 5'(GAATTCAGC AAGTTCAGCCTGGTTAAGT)3' and reverse 30mer 5'(TACCCTGGAAGAATACTCATA AGGAATTC)3' primers (Iwaki Glass Co, Ltd., Chiba, Japan). The PCR products were then ligated into T vector (Amersham International plc, Bucks, United Kingdom), and the recombinant plasmid DNAs were purified to analyze DNA sequences. In the process of these screenings and characterizations, the nucleotide sequence data showed that a clone named LL55 with a 331 base pair (bp) insert encodes a putative RNP which is expressed selectively in lymphoid lineage cells. Using the 331 bp probe,

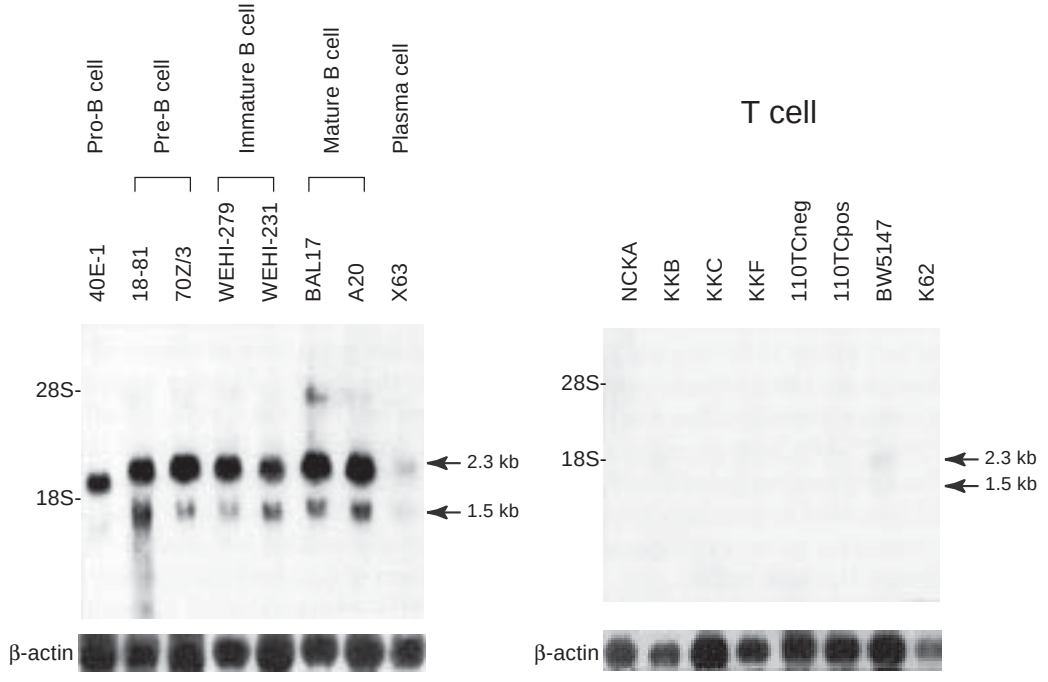

Fig. 1. Northern blot analysis on various lymphoid cell lines. Total RNAs [20 μg (B cell lines) and 10 μg (Tcell lines)] extracted from various lymphoid cell lines as listed in Table 1 were separated on denaturing agarose gel and the transferred filter was hybridized with a LL55 cDNA probe. To show the amount of mRNA loaded, a second hybridization of the same filter was performed using human β-actin cDNA as indicated. LL55 mRNA appears as 2.3 and 1.5 kb bands (arrows).

Table 1. Characterization of lymphoid cell lines used for Northern blot analysis

Lineage	Cell line	Developmental stage	Ig or TCR rearrangement		Surface expression			
B cell	40E1	Pro-B	Heavy chain: DJ Light chain: N		Ig(–)			
	18.81 70Z/3	Pre-B	Heavy chain: VDJ Light chain: N		μ/VpreB/l5			
	WEHI-231 WEHI-279	Immature B	Heavy chain: VDJ Light chain: VJ		$IgM^{high(+)}/IgD^{low(+)}$			
	BAL17 A20	Mature B	Heavy chain: VDJ Light chain: VJ		$IgM^{low(+)}/IgD^{high(+)}$ IgG(+)/IgD(+)			
	X63	Plasmacyte			Ig(–)			
			α	β	CD3	CD4	CD8	Thy1
T cell	NCKA	Double negative	Partially rearranged	Not rearranged	–	–	–	+
	BW5147 KKB KKC 110TCneg	Double negative	Rearranged	Partially rearranged	–	–	–	+
	K62 KKF 110TCpos	Double positive	Not rearranged	Rearranged	+	+	+	+

μ/VpreB/λ5, pre-B cell receptor.

another fetal (day 11) cDNA library constructed in λgt11, kindly provided from Dr. Seiji Miyatani, was further screened to find the full length clones. To determine the nucleotide sequence of the 5' side of the mRNA, the rapid amplification of 5'-ends of cDNAs (5' RACE) was performed according to the method described by Frohman and coworkers (1988).

Northern blot analysis

Total RNAs from various lymphoid cell lines were purified using Polytron (Kinematica AG, Lucerne, Switzerland) with the reagent of ISOGEN-LS (Nippon Gene, Toyama, Japan). Northern blot analysis was performed by the method of 3-(*N*-morpholino)propanesulfonic acid (MOPS)/formaldehyde/agarose (Sambrook et al., 1989), and was transferred onto Hybond-N+ filter (Amersham). The filter for fetal RNAs and the tissue RNAs was purchased from CLONTECH Laboratories.

Results

By differential screening of the fetal liver (day 10.5) cDNA library, we isolated a cDNA clone with a 331 bp insert that is expressed in lymphoid lineage cells. Using this LL55 probe, Northern blot analysis on various cell lines including pro-B, pre-B, immature B, mature B cell lines, plasmacytoma and T cell lines demonstrated the 2.3 kilobase (kb) RNA transcript in the lymphoid cell lines (Fig. 1, Table 1). Using mRNAs from normal organs or tissues, LL55 mRNA appears as 2.3 and 1.5 kb

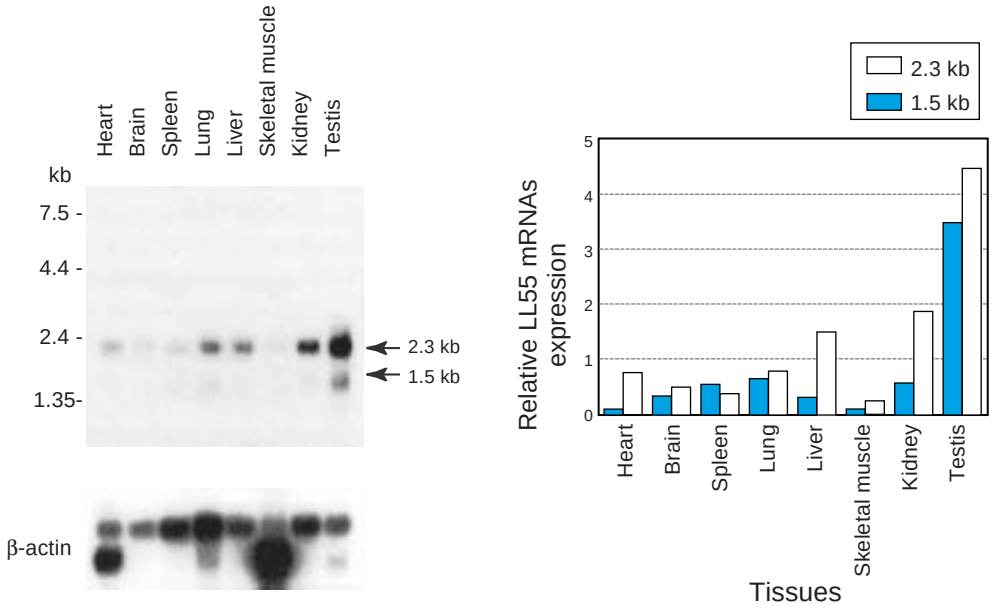

Fig. 2. Northern blot analysis on normal organs or tissues. **A:** Northern blot containing approximately 2 μg of poly $(A)^+$ RNA per lane from eight different mouse tissues. It was purchased from CLONTECH Laboratories. A second hybridization of the same filter was performed using human β-actin cDNA. **B:** The expression of LL55 mRNAs was compared among normal organs or tissues by the densitometric scanning of the Northern filter. Signals for two different sizes of LL55 mRNA were compared based on the expression of β-actin.

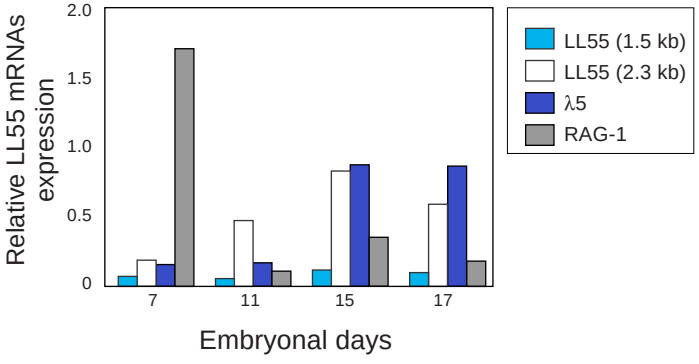

Fig. 3. Expression of LL55 mRNA during the development of mouse embryos. Northern blot containing approximately 2 μg of poly $(A)^+$ RNA per lane from mouse embryos of different developmental stages as days 7, 11, 15 and 17. It was purchased from CLONTECH Laboratories. The filter was stripped of radioactivity and hybridized with a radioactively labeled [α-^{32}P]dCTP mouse λ5, mouse RAG-1 and human β-actin cDNA control probe. Relative amounts of each λ5, RAG-1 and LL55 specific mRNA species were estimated by densitometric analysis. Signal intensities were normalized on the basis of the β-actin signals for each sample.

bands (Figs. 2A and B). LL55 is expressed in the heart, spleen, lung, liver, kidney and the testis, but the expression is weak in the skeletal muscle and the brain. The expression of LL55 mRNA was most abundant in the testis by our Northern blot analysis. The relative expression of two forms of LL55 mRNA was compared based on the expression of β-actin (Fig. 2B). LL55 mRNA from the heart, liver and the kidney is predominantly 2.3 kb, while the other organs express similar amounts of mRNA with both sizes.

The expression of LL55 mRNA was studied during the development of lymphoid organs in the fetal life. The

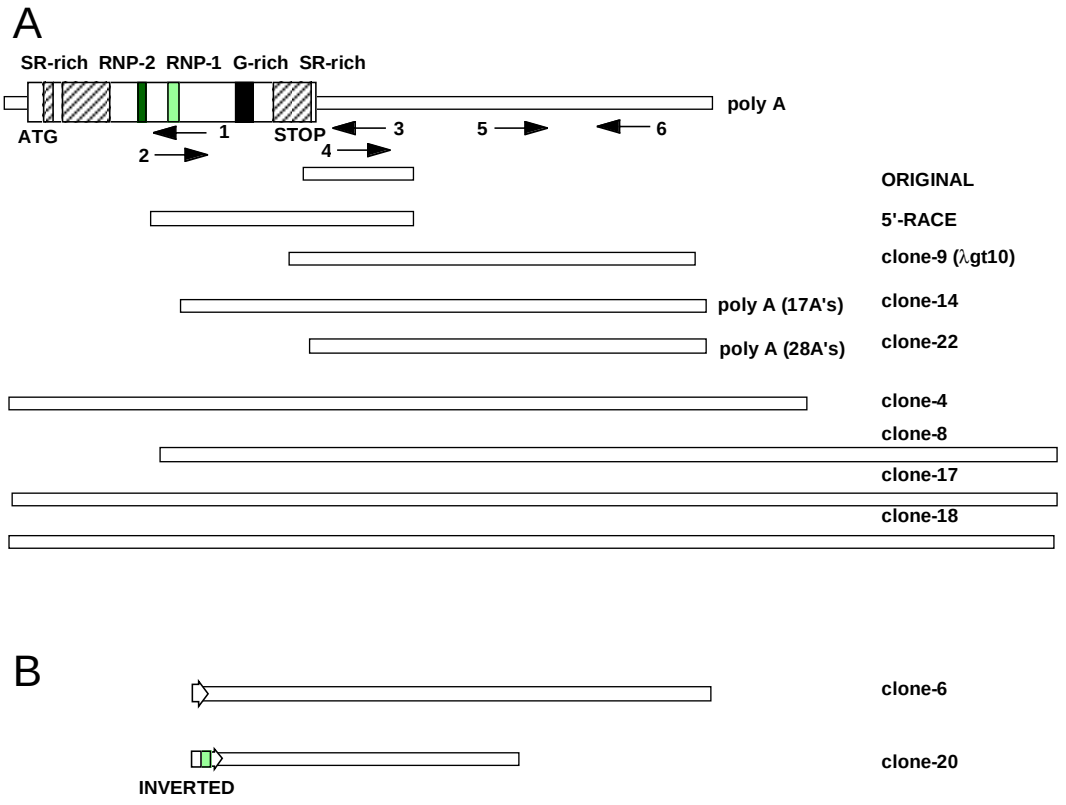

Fig. 4. Relative map of the LL55 clones. **A:** Relative map of the clones isolated using the original (331 bp) cDNA probe. Only clone 5'-RACE was isolated by the 5'-RACE method. Narrow open boxes indicate the consensus sequence of clone-17. Wide open boxes indicate the coding region of LL55 polypeptide (288 amino acids) with RNP-1 domain (□), RNP-2 domain (■), SR-rich domain (▨) and G-rich domain (■). The longest clone clone-17 contains the 4 kb insert. Clone-14 and clone-22 have polyadenylation signal and poly A. **B:** Clone-6 and clone-20 contain inversions of their sequences (open arrow box). Clone-20 contains an inversion of the RNP-1 domain.

relative expression of the transcripts is shown based on the expression level with β-actin probe for the control of RNAs. LL55 mRNA appears with the sizes of 2.3 kb (□) and 1.5 kb (■) at day 11 and is up-regulated during fetal development, reaching the maximum at day 15 (Fig. 3). Markers for lymphoid generation were also monitored on the same blot as RAG (recombination activating gene)-1 (■) for the gene rearrangement of the antigen receptor genes (Oettinger et al., 1990), and λ5 (■) for pre-B cell generation (Sakaguchi and Melchers, 1986). LL55 expression is up-regulated in parallel with the expression of λ5 mRNA.

Using the LL55 cDNA probe, the longer cDNA clones were isolated and their sequences were determined respectively. Figure 4A shows the relative map of the clones. One of the clones named clone-17 spans for a possible longest reading frame. To detect the alternative splicing of LL55 RNA, the PCR method with several combinations of primers was performed for the analysis of cDNA clones isolated in the schematic orientation (Fig. 4A). By Northern blot analysis, the most abundant class of mRNA is 2.3 kb RNA with all four kinds of domains such as 5' SR, RNP-2, RNP-1, G-rich and 3' SR. The next class contains G-rich and 3' SR

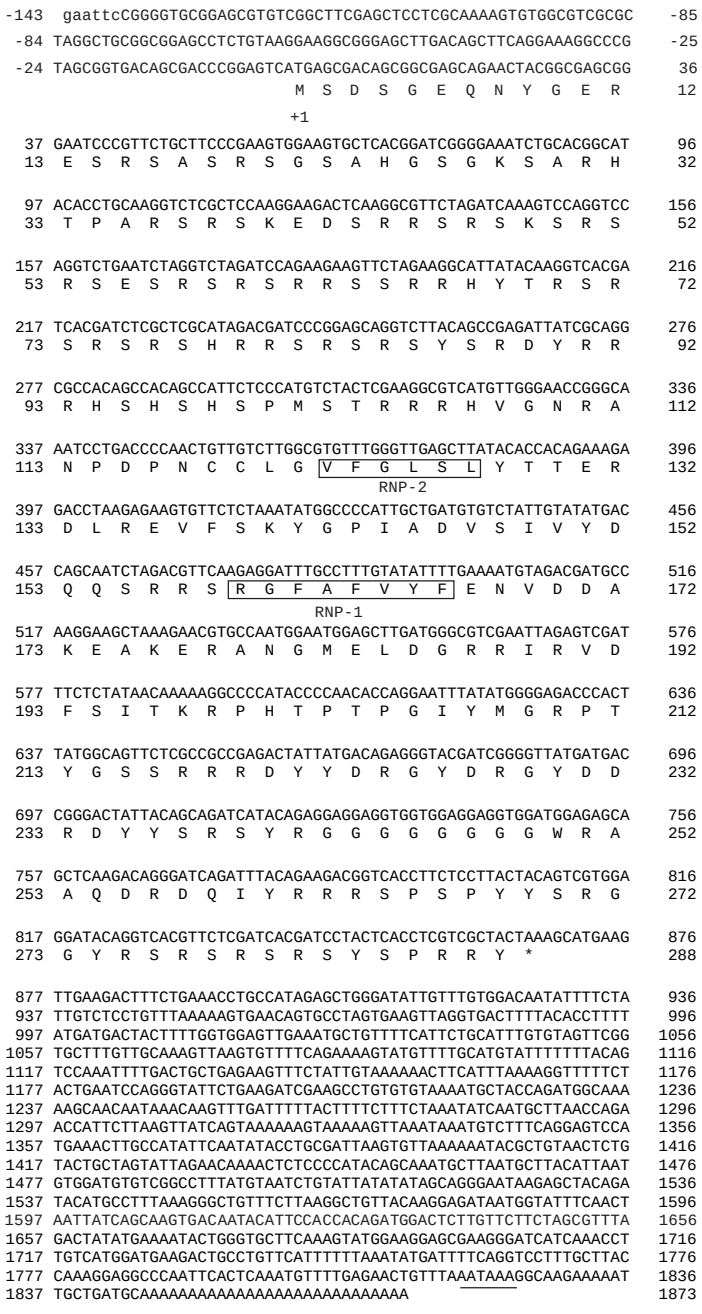

Fig. 5. Nucleotide and amino acid sequence of LL55. Homologous stretches for RNP is indicated by an open box for RNP-1 and RNP-2. The poly adenylation signal AATAAA is indicated by an underline.

domain, but lacks 5' SR, RNP-2 and RNP-1 (data not shown). These two forms are also confirmed with cDNA clones isolated using 5' RACE method (Fig. 4A; clone 5'-RACE) (sequence data not shown). By screening a fetal (day 11.5) cDNA library with the LL55 probe containing RNP-1 domain, we obtained 33 clones with different sizes of insert DNAs. Analysis of two clones with 4.2 kb inserts demonstrated that both clones contain an identical sequence but several clones show the inversion of the RNP-1 domain, creating a truncation of protein coding (Fig. 4B; clone-6 and clone-20) (data not shown). These results suggest that the expression of the LL55 protein might be also controlled under alternative RNA splicing.

Figure 5 shows the nucleotide and the amino acid sequences of LL55. There is a poly-A sequence on one end with a polyadenylation signal AATAAA located 28 bp upstream, determining the orientation of the cDNA clone. The open reading frame encodes a putative protein with 288 amino acids starting from the methionine at the nucleotide number 143 and ending as TAA at the nucleotide number 865. The first methionine codon does not match the consensus sequence for optimal initiation (Kozak, 1987). The nucleotide sequence of this clone showed the homology to a known RNP of Drosophila, the sex determining protein of transformer-2 (tra-2) (49.2% identity in nucleotide sequence and 38.2% in amino acid sequence) (Belote and Baker, 1983; Amrein et al., 1988, 1990). And a part of the nucleotide sequence of this clone corresponds to the SIG41

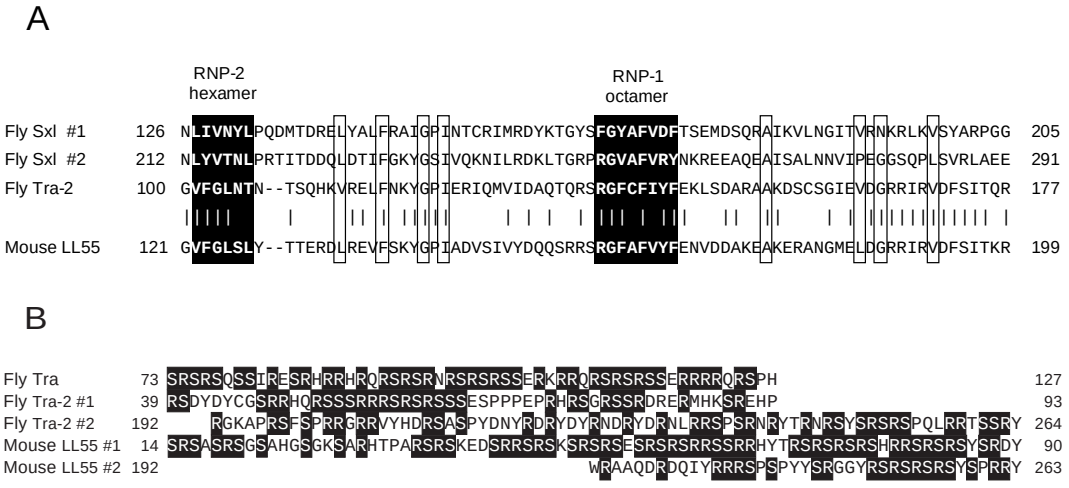

Fig. 6. **A**: Alignment of LL55 protein RNP. Conserved features among RNP domains of LL55 and other proteins. Regions of sequence similarity among several SR-proteins and LL55 are arranged for the best alignment of conserved residues within sub-domains of RNA binding motif. The RNP-1 and RNP-2 elements are shown in reverse type, and eight other highly conserved positions are show in narrow open boxes. The numbers on the left and right ends of the sequence indicate the positions of each SR proteins in the respective amino acid sequences. **B**: Alignment of SR-rich domain of LL55 protein. Comparison of SR domains of LL55 to SR domains of SR-protein family. Amino acid identities and semiconservative changes are indicated by shading.

gene that is induced by silica treatment in murine macrophage cells and is homologous to the RA301 gene that is induced by hypoxia/ reoxygenation in rat astrocytes (Matsuo et al., 1995; Segade et al.,1995). The putative LL55 protein contains apparent consensus motifs for the RNA-binding protein with highly conserved oligopeptide cores called RNP-1 (octapeptide) and RNP-2 (hexapeptide), both of which are surrounded by the serine-arginine repeats (SR domain) spanning to 100 amino acids in the amino terminal and to 50 amino acids in the carboxyl terminal regions (Figs. 6A and B). This protein also has a glycine-rich domain including 8 glycine residues. It may act as a hinge region between N-terminal and C-terminal domains. The proteins with RNP and SR domains are classified as the SR-protein family, whose function is essential for RNA splicing. Compared with a number of SR proteins in *Drosophila melanogaster*, mouse, rat and human, LL55 contains two RNA-binding domains in the middle of the protein and SR domains in both amino- and carboxyl-terminals (Fig. 7)

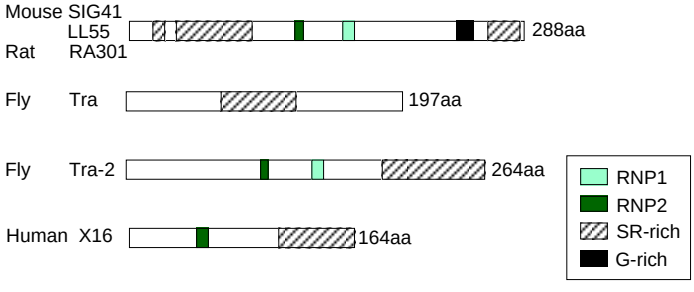

Fig. 7. Diagram of the SR family proteins. Overall structure of LL55 polypeptide and several RNP proteins are compared based on the key amino acids commonly seen among the members of SR-protein family. Various domains represented include RNA binding motif with internal octapeptide RNP-1 core (light shaded box), hexapeptide RNP-2 core (dark shaded box), SR-rich domain (diagonally striped box) and glycine-rich domain (black box).

Discussion

In the present study, we isolated a cDNA clone expressed in fetal development which potentially encodes an RNP with RNA splicing activity. The putative protein of LL55 has RNP- and SR-domains and classified as an SR protein family. In the initial screening, it was expressed specifically in the lymphoid lineage cells. By using the specific probe which does not cross-react with other members of the SR family, LL55 is expressed ubiquitously in many kinds of organs and tissues as shown in Figs. 2A and B, but is preferential in the lymphoid organs and the testis. Interestingly, LL55 mRNA appears in fetal development as early as day 11 of gestation when lymphoid generation has just occurred (Fig. 3), especially in the fetal liver detected by the in situ RNA hybridization method (data not shown). This expression profile suggests that LL55 RNP might play critical role in the generation of early lymphoid precursor cells.

Alternative splicing is one of the main mechanisms including other gene duplications and gene rearrangements that evolve to produce protein diversity at the level of the primary structure. Several reports concerning the tissue specific expression of human or murine RNPs provide information about physiological function in the development and activation of eukaryotic cells (Vellard et al., 1992; Diamond et al., 1993). The gene rearrangement of Ig and TCR genes is an efficient mechanism for the generation of protein diversity from a limited number of genes. It irreversibly changes the genetic content of the cells, committing the cells to a process of terminal differentiation. Alternative splicing offers many advantages. It does not change the genetic content of the cell nor is it irreversible. Splicing pathways need not be discarded to use one of the molecules, creating a stable diversity during early developmental stages.

In the immune system, however, it remains to elucidate the expression and the function of lymphoid specific RNPs regulating RNA splicing of a variety of lymphoid specific molecules during development. Previously, Ayane and coworkers (1991) reported a gene named X16 encoding a protein with a nucleotide binding motif, which is expressed differentially in human pre-B cells (Fig. 7). Expression of LL55 mRNA is more restricted to the earlier stages of the lymphoid development than X16 gene. Lymphoid RNPs could play roles at various phases in the development of the immune system. Expression of Ig and TCR genes probably requires a specific RNA splicing process allowing the expression of a functional J_H sequence without the co-expression of 3'-J_H sequences (Hiramatsu et al., 1995). Recently, Aoufouchi and coworkers (1996) demonstrated that the intronic sequence influences κ gene RNA splicing in a cell-free system, suggesting the important role of RNA splicing in the regulation of Ig expression during the development of B lineage cells. Differential RNA splicing events in the lymphoid system are reported: the activation of antigen receptor positive B cells switches the pattern of RNA splicing from a membrane form to a secreted form in Ig mRNAs (Early et al., 1980); lymphoid differentiation accompanies lineage specific RNA splicing of CD45 as CD45RO for B cells and CD45RA, RB, RC for T cells (Screaton et al., 1995). Alternative RNA splicing and production of different mRNAs leads to co-expression of μ and δ or membrane and secreted immunoglobulin, and co-expression of multiple heavy chain clones in individual B cells. Alternative RNA splicing plays an important role in the immune system.

From Northern blot analysis LL55 mRNA appears in sizes 2.3 and 1.5 kb during the embryonal stage, although the transcript of the LL55 gene varies during the development. A number of LL55 clones were isolated from the fetal cDNA library and their sizes were studied by restriction mapping and PCR analysis using several combinations of LL55 primers. One of the clones showed the apparent inversion of the RNP-1 domain (Fig. 4B; clone-20), which probably caused the defective function in RNA splicing activity. It has been often shown that many SR proteins show regulatory function by

alternative splicing of their own RNA molecules to create a truncated form of mRNA (Bingham et al., 1988). Although our results did not clearly demonstrate the function of the SR protein in the LL55 molecule for the development of lymphoid cells, the alternative form of mRNA was shown by Northern blot analysis and the RT-PCR method. Especially, the inverted form of LL55 would be a sign for the RNA splicing function of the LL55 molecule. We tried to determine a protein molecule encoded by LL55 using the in vitro transcription and translation method. The protein molecule synthesized from the LL55 cDNA clone introduced into pBluescript vector showed an apparent molecular weight of 35 kDa, 30 kDa and 19 kDa on SDS-PAGE analysis (data for the paper prepared by Dr. Hideya Igarashi and others). It will be necessary in the future to study the RNA splicing function of the RNA molecules used in the differentiation and activation of lymphoid cells.

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