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Expression of the Proliferation-Related Ki-67 mRNA in the Early Development of Murine Embryo

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In a search for early lymphoid-specific genes, we isolated a cDNA clone (LL7) encoding a murine homologue of Ki-67 protein, a proliferation-related nuclear antigen. LL7 transcript appears preferentially in lymphoid organs as the bone marrow, spleen, and the thymus. Here, we studied the expression of murine Ki-67 (mKi-67) mRNA among various organs or tissues during the early development of fetus. In fetus, mKi-67 mRNA appears developmentally as early as day 11 and is expressed maximally at day 15. In situ hybridization on the section revealed that the expression of mKi-67 mRNA is preferential in the area of active organ formation such as neurological system and the fetal liver. These results suggest that mKi-67 plays an important role in the proliferation of early embryonic precursor cells of neurological and immune systems.

Lymphoid precursor cells are generated in the fetal life as early as day 10.5 from the blood islet of mesoderm origin and maturate with multiple molecular events for the differentiation and proliferation to induce the rearrangement of immunoglobulin or TCR genes (1-3). Fetal liver is a first embryonic organ which supports the development and the rapid proliferation of lymphoid precursor cells to generate sufficient number of cells for the primary repertoire of antigen specificity (4-6). Cell proliferation of embryonic stage is probably controlled by a different mechanism from that of somatic cells. The early embryonic cell cycles differ significantly from the cell cycles of most somatic cells. They are very rapid and consist of altering S and M phases without intervening G1 and G2 phases. The key regulatory steps of the somatic cell cycle are absent and the duration of S phase is shorter as 25 min than approximately 8 hrs in the somatic cell cycle (7). Several transcription factors such as Ikaros, BSAP, Ets-1, c-Myb, EBF, E2A and GATA3 are involved in the generation and proliferation of lymphoid precursor cells (8-14), however their target molecules for the cell cycle control during embryonic stage is not clearly determined. To understand the molecular mechanism of the proliferation of lymphoid precursor cells, we tried to isolate genes that are expressed preferentially in the early fetal development around day 10 and day 13.

A cDNA clone named LL7 is expressed as early as day 11 and is up-regulated at day 15 and then gradually decreased at day 17. The expression profile in the fetal life seemed to be parallel to the stages of the active lymphoid formation in the fetal liver. Nucleotide sequence analysis revealed that the LL7 cDNA is homologous to the gene of human proliferation-related nuclear antigen Ki-67 (15). In human and mouse, Ki-67 protein is localized in the nucleolar cortex and is preferentially expressed in proliferating cells (15-17). The expression of Ki-67 protein is an indicator for the proliferative activity of the cells both in vivo and in vitro (18, 19). Micro injection of anti-murine Ki-67 specific antibody causes a delay in cell cycle progression in vitro (18). Anti-sense oligonucleotide of human Ki-67 shows an inhibition for thymidine-incorporation in IM-9, a multiple myeloma cell line (15). These experiments indicate that Ki-67 protein takes part in an essential cell cycle-related process in the mature lymphoid cells. Therefore, it is also considered that Ki-67 is involved in the proliferation of early embryonic cells.

Here, we studied the expression of mKi-67 transcript in the early embryonal life by the in situ RNA hybridization and demonstrated that the message is preferentially expressed in the area of neurological tissues as forebrain, midbrain, telencephalon, dienchephalon, the neural canal, and the fetal liver. The expression is re-

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markable in the fetal liver as early as day 11.5 to 13.5. These results would provide an important information for the understanding of mKi-67 in the induction of the proliferation and differentiation of lymphoid precursor cells.

MATERIALS AND METHODS

Mice. Balb/c mice were purchased from Japan Crea Co. (Tokyo) and maintained in the Animal Center of Kumamoto University or Medical Center of Chiba University Medical School.

Cells and cell culture. Lymphoid cells from the spleen, thymus, and bone marrow were obtained from 6-week old Balb/c mice. All the cells were cultured in the culture medium of RPMI-1640 (Life Technologies, Inc., Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS) (M. A. Bioproduct, Walkersvile, MD), 2 mM L-glutamine, 2-mercaptoethanol (5×10^{-5} M), 100 μg/ml of streptomycin, and 100 μg/ml of penicillin (Wako Co, Osaka, Japan). Cells were cultured at 37 °C in 5% CO_2 incubator.

Cloning of LL7 cDNA clone. A lymphoid specific cDNA clone (LL7) was isolated from the murine fetal cDNA library in λgt-10 phage (day 11.5 fetus; CLONTECH Lab, Inc, CA) as the tissue and stage-specific gene. The nucleotide sequence of the insert DNA revealed that the gene is a murine homologue of human Ki-67 gene encoding a proliferation-related nuclear antigen (15). The entire sequence of the murine gene was reported previously (16).

Northern blot analysis. The murine Ki-67 expression was detected with the probe of 700 bp insert corresponding to the nucleotide numbers from 3,714 to 4,375 of the murine Ki-67 gene (16). RNA blot filters were prepared in our laboratory by the method described previously (20). For several fetal RNA samples, we used filters from the company (CLONTECH). To study lymphoid specific mRNAs, RAG1 cDNA was prepared by RT-PCR method using primers (5′-AACAGATGTCACAGGACGGT-3′, 5′-GTAGCTTAGCCAACATGGCT-3′) and the 700 bp insert subcloned in pGEM-T vector (Promega, Madison, WI) was confirmed by sequencing (21). To detect λ5 mRNA, a 700 bp λ5 cDNA insert was used (22). All probes were radiolabeled with α-[^{32}P]-dCTP (Amersham, Arlington Heights, IL) using random primer radiolabeling kit (Takara Biomedicals, Tokyo). All the filters were re-probed with the control β-actin (CLONTECH) probe to confirm the amount of RNA loaded in each lane.

In situ hybridization. In situ hybridization was performed as described previously (23). Single stranded RNA probe containing [^{35}S]-uracil (Amersham) was synthesized from linearized LL7 template cDNA according to the manufacture's protocol (Promega). Embryos of day 7.5, 11.5 and 13.5 were dissected out and fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffer saline (PBS) and embedded in paraffin wax (Sigma, St Louis, MO). Sections were cut for slices at 8 mm and placed onto TESPA (Sigma)-coated slides prepared as described (23). The sections were dewaxed in xylene, rehydrated with ethanol, treated with 20 mg/ml of proteinase K (Boehringer Mannheim Gmbh., Mannheim, Germany), acetylated (1.25% triethanol amine, 0.25% acetic anhydride) and dehydrate with ethanol. Then the sections were hybridized with [^{35}S]-uracil labeled riboprobes (1×10^{8} cpm/ml) at 50 °C overnight in the hybridization buffer [50% formamide, 2 mg/ml PVP (Sigma), 2 mg/ml Ficoll 400 (Pharmacia, Uppsala, Sweden), 10 mM NaH_2PO_4 pH6.8, 5 mM EDTA, 0.3 M NaCl, 10 mM Tris pH 8.0, 0.1 M dithiothreitol (Sigma), 10% Dextran sulfate (Pharmacia)]. The hybridization mix was boiled, applied directly onto the sections, and covered with siliconized cover slips. The sections were washed with 2×SSC, 50% formamide, 1.4 mg/ml 2-mercaptoethanol at 37 °C for 15 min, then at 65 °C for 30 min, and incubated in NTE (0.5 M NaCl, 10 mM Tris, 5 mM EDTA) containing 20 μg/ml RNase A (Sigma) at 37 °C for 15 min. Subsequently the sections were washed, dehydrated with ethanol, and dried up in the air. The sections were dipped in Kodak NBT emulsion (Kodak Japan Ltd., Tokyo) (diluted 1:1 with 2% glycerin), exposed for two weeks and developed in Kodak D-19 solution.

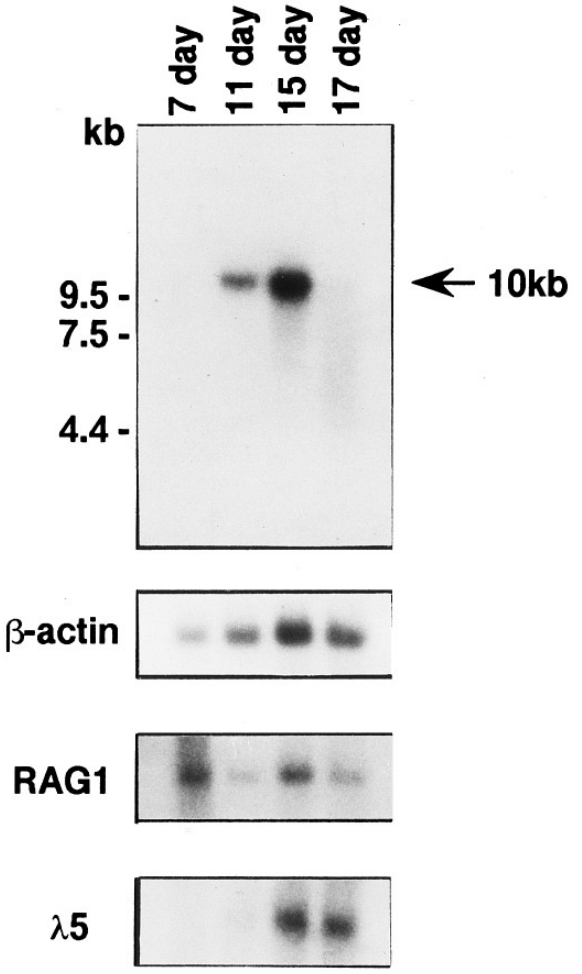

FIG. 1. Alteration of LL7 transcripts during murine embryogenesis. Northern blot analysis with 700 bp fragment of LL7 was performed onto the MTN membrane (CLONTECH). The transcripts of LL7 appear at 10 kb length on the membrane indicated by an arrow. The same membrane was deprobed, and then reprobed with β-actin as the control, with RAG1 and with λ5 for the monitoring of lymphopoiesis.

RESULTS

Developmental expression of mKi-67 transcripts in the fetal life. From the cDNA library of the day 11.5 fetus, we isolated a cDNA clone (LL7) of 700 bp insert by the differential screening with two kinds of mRNAs from adult and fetal livers. The expression of LL7 mRNA varies during the development of the fetus (Fig. 1). The peak expression, in comparison to the signal with β-actin probe, appears at day 15, which is quite similar to the expression of the genes related to the

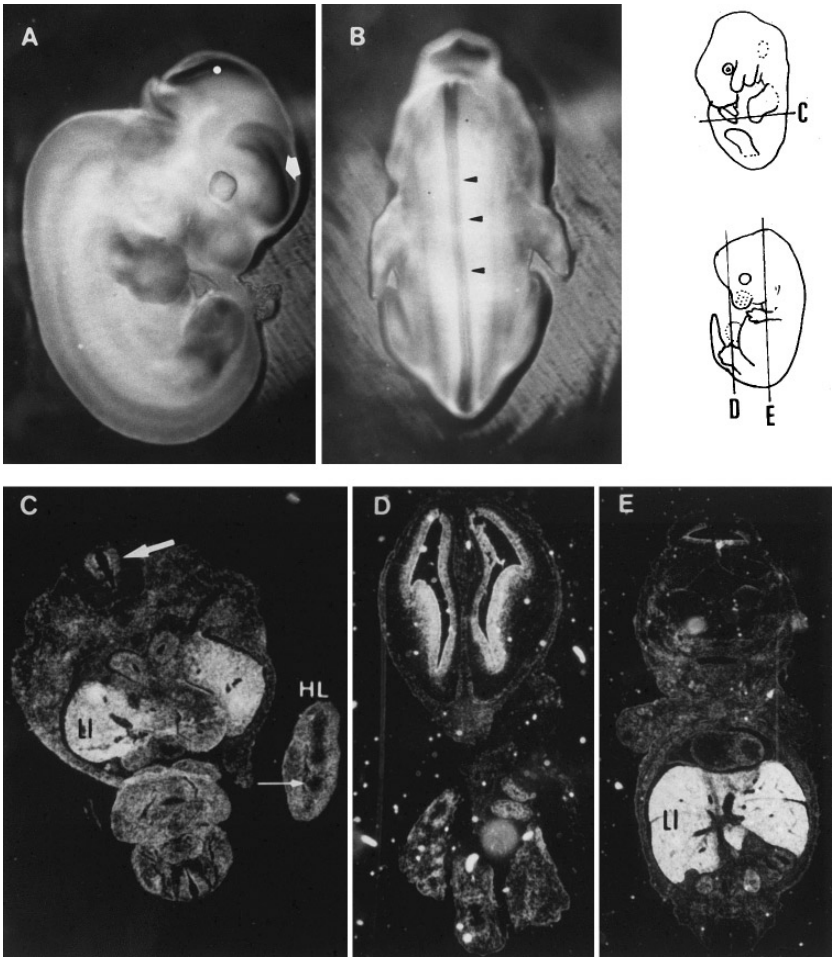

FIG. 2. In situ localization of mKi-67 in murine fetus. Distribution of mKi-67 transcripts was investigated by in situ hybridization in embryos of day 11.5 (A-C) and day 13.5 (D, E). In panel A, an arrow and an asterisk indicate the forebrain and the midbrain, respectively. Both regions show high expression of mKi-67. Accumulation of mKi-67 is also seen at the spinal cord along the entire axis which is indicated with arrow heads in panel B and bold arrows in panel C. A thin arrow in panel C demonstrates the absence of mKi-67 in the region containing mesenchymal cells of limb bud development. LI indicates the fetal liver and HL shows the hindlimb.

lymphoid cell generation such as RAG1 and λ5 (21, 22). The RAG1 probe also shows an apparent band at day 7, showing the biphasic expression during embryogenesis. The RAG1 mRNA appears probably in the precursor stages of lymphoid lineage cells. The λ5 protein is a surrogate light chain expressed selectively in the pre-B cell stage. The appearance of λ5 mRNA is followed with the expression of LL7 mRNA (lowest panel). The nucleotide sequence of the LL7 cDNA shows 57% homology to human Ki-67 gene (data not shown). The LL7 cDNA shows a stretch of 97% homology to the sequence of murine Ki-67 cDNA submitted to EMBL data base during our processing.

Expression of mKi-67 mRNA during embryonic development of mice. The mKi-67 mRNA is expressed as early as day 7.5 of the embryo as detected by the in situ hybridization method on the whole mount of the embryo. Figure 2A and 2B show that mKi-67 mRNA is highly positive in the primoderium for the neurological development, especially in the forebrain (Fig. 2A; pointed by an open arrow) and the midbrain (Fig. 2A; pointed by an open dot). It is expressed as the dark signal in the very restricted region of the neural canal distributed from the invasion site to the tail. The pecuriality of mKi-67 expression is the loss of the expression in the central area of the neural canal (Fig. 2B; pointed by solid arrow heads), whose expression suggests that mKi-67 expression is selective in the area of the precursor cells for the neurological development. The mKi-67 mRNA is also abundant in the area of primoderium for the hands and pedis, with the clear hollow in the middle. On the section of day 11.5 and day 13.5 embryo, the bright signal of mKi-67 mRNA is detected as well in the primordial region for the neurological development of the neural canal (Fig. 2C: pointed with a large open arrow) and the lateral ventricles (Fig. 2D). Fetal

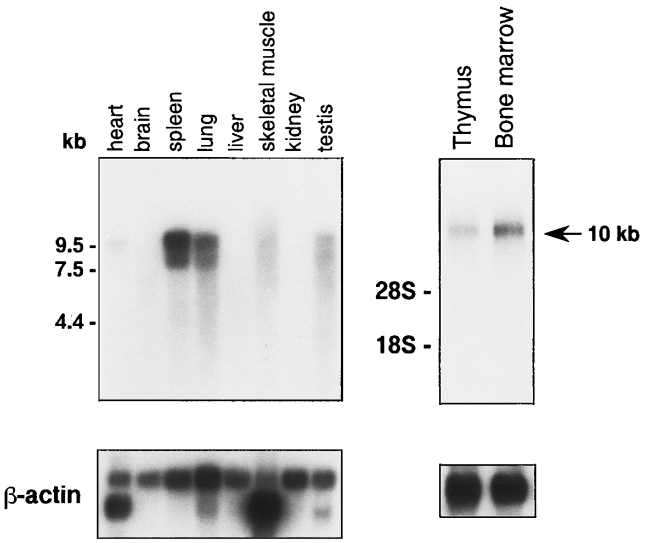

FIG. 3. Tissue distribution of mKi-67 in adult mice. Northern blot analysis was conducted on the membrane with RNAs isolated from various kinds of organs of adult mice. The autoradiogram revealed a band of 10 kb indicated by an arrow. Hybridization of the blot with β-actin cDNA was used as the control of RNAs loaded. The mKi-67 mRNA is expressed preferentially in lymphoid organs.

liver most abundantly expresses mKi-67 mRNA at day 11.5 and day 13.5. The expression is very dense throughout the fetal liver as clearly demarcated by the sinusoid configuration. These results suggested that the expression of mKi-67 mRNA is very selective in the area of the cells that have started the proliferation in the embryo.

Murine Ki-67 mRNA is preferentially expressed in the lymphoid organs. The selective expression of mKi-67 in the fetal life suggested that Ki-67 is closely-related to the activation and differentiation of lymphoid cells. Northern blot analysis demonstrated that mKi-67 mRNA is expressed preferentially in the adult spleen and lung, but it is expressed at the lesser extent in the heart, skeletal muscle, and the testis (Fig. 3; left panel). Although the signals from the heart, skeletal muscle, and the testis are very weak, there are apparent 10 kb bands in the original photograph. It was not detected in the brain, liver, and the kidney. The mKi-67 is also expressed in the bone marrow and the thymus (Fig. 3; right panel). The cells in these lymphoid organs are in the mitogenic state and a numerous number of lymphocytes are generated as well as from a fetal liver. The result indicates that the expression of mKi-67 is correlated to the proliferative activity of cells in adult mice.

DISCUSSION

The mKi-67 mRNA is observed as early as day 11.5 in the very specified organs such as neurological and immunological systems. In situ hybridization suggested that the expression pattern of the gene is similar to the area of the bromodeoxiuridine (BrdU) incorporation (a derivative of thymidine) (24, 25). High levels of mKi-67 mRNA are expressed in the proliferating cells that show a marked incorporation of BrdU. The signals to induce the marked expression of mKi-67 mRNA would be involved in the growth promotion in the embryonal life.

The expression of mKi-67 mRNA in mice resembles to the nuclear transcription factor Pax-5 that is expressed in the early development of the embryo (9). The mRNAs of both genes appear at the maximum level during day 10 and day 15 of gestation. They are observed in the similar areas associated with the formation of nervous system and of lymphoid system (fetal liver). Table 1 shows a comparison of mKi-67 expression with that of Pax-5 during embryonic development detected by Adams et al. (9). Strong expression of mKi-67 is observed at the neural regions containing proliferative cells, especially in the ventricular zone and mantle layer. The neural stem cells are newly synthesized from ventricular zone and the neurons achieved a dif-

TABLE 1

Similarities of Expression in Mice between mKi-67 and Pax-5

		mKi-67	Pax-5[a]
Gestation	Day 7	±	±
	Day 11	+	++
	Day 15	++	+
	Day 17	±	+
Fetal 11.5 D[c]	Prosencephalon	+	ND[b]
	Mesencephalon	+	+
	Metencephalon	ND	+
	Telencephalon	ND	+
Fetal 13.5 D	Liver	++	+
	Ventricular zone	+	+
	Mantle layer	+	+
Adult 6 W[d]	Haemato-lymphoid organ		
	Spleen	++	++
	Bone marrow	++	ND
	Thymus	++	ND
	Lymph node	ND	–
	Blood	ND	++
	Non-lymphoid organ		
	Lung	++	++
	Brain	–	++
	Heart	±	–
	Kidney	–	–
	Liver	–	–
	Testis	+	++

[a] The expression of Pax-5 referred from Adams *et al.* (9).
[b] Not determined.
[c] Day of fetal life.
[d] Week after birth.

ferentiation from the stem cell move to the mantle layer in the neural canal. Pax-5 appears in fetal liver at day 13.5 and the expression gradually increases during the development. The mKi-67 mRNA appears in the fetal liver at day 11.5 and it becomes at maximum level 2 days later. This expression profile seems to be in accordance with the lymphopoiesis of the fetal liver. The B cell development of Pax-5 deficient mice were impaired at the stages from large pre-B to small pre-B cells, suggesting a critical role of Pax-5 in the B cell development. Pax-5 deficiency also causes abnormalities in the neurological development such as the disappearance of the central region in the dorsal midbrain (26). Similar expression profiles of Pax-5 and mKi-67 mRNAs by in situ hybridization suggest a specified role of mKi-67 during the early embryonal neurogenesis and lymphopoiesis.

Although the expression of mKi-67 mRNA is ubiquitous in various organs and tissues, it is preferentially expressed in the adult spleen and lung. The high expression of mKi-67 in the lung is probably explained by the presence of haematopoietic cells circulating in the lung. The expression profile of mKi-67 mRNA in adult organs is similar to that of Pax-5 except for thymus (Table 1). Several experiments, using the antisense oligonucleotide or the antibody, showed that Ki-67 is actually involved in the regulation of cell proliferation (15,16). Crosslinkage of antigen receptor on B cells induces the accumulation of B cell specific transcription factor (BSAP) encoded by Pax-5 gene as well as the incorporation of [^{3}H]-thymidine (27). The antisense oligonucleotides of Pax-5 also inhibits the proliferation of spleen B cells stimulated with lipopolysaccarides, suggesting that Pax-5 and Ki-67 are involved in the similar mechanism of the cell proliferation. The absence of Pax-5 in the thymus might implicate that the promoter of mKi-67 is similar but is different from that of Pax-5. Since Pax-5 is known to regulate the expression of several functional molecules involved in the B cell development, it is necessary to determine whether the expression of mKi-67 mRNA is controlled by Pax-5 during embryonal development.

In spite of a number of reports concerning to Ki-67, only a little is known about the molecular mechanism of this molecule. The mKi-67 protein is a 325 kDa nuclear protein, calculated from deduced amino acid sequences, with the multiple repeat structure of Ki-67 repeat (Ki-67 motif) and is expressed in the nuclei of proliferating cells of various organs. There are a number of putative phosphorylation sites as 125 sites for protein kinase C and 54 sites for casein kinase II, potentially providing the ability for the protein/protein interaction of this macromolecules in the nuclei. There are two putative nuclear localization sequences. LL7, a partial fragment we isolated, corresponds to the mKi-67 at the position of 3,685-4,391 bp containing the second Ki-67 motif out of 13 motifs in the entire mKi-67 amino acid sequence (16). This motif contains several Thr-Pro-X-X sequence. Suzuki suggested that the Thr-Pro-X-X sequence forms a β-turn I structure stabilized by two hydrogen bonds and posses a DNA-binding ability. The Thr-Pro-X-X sequence is widely found in various transcription factors containing Zn finger or helix-turn-helix structures (28). Thus it is suggested that mKi-67 might be involved in the cell proliferation by binding to the upstream region of various genes and the regulatory molecules for the gene expression. Based on this consideration, it would be necessary to study the molecules regulated by mKi-67 during the embryonal lymphoid development.

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