# Cell Cycle Control of c-*kit*$^+$IL-7R$^+$ B Precursor Cells by Two Distinct Signals Derived from IL-7 Receptor and c-*kit* in a Fully Defined Medium

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## Summary

An important goal for the investigation of the proliferation of mammalian cells is to establish a fully defined condition for culturing them in vitro. Here, we report establishment of a fully defined culture condition that supports the primary culture of normal c-*kit*$^+$IL-7 receptor (IL-7R)$^+$ B precursor cells without the aid of stromal cell lines. This defined culture condition contains IL-7, the ligand for c-*kit*, transferrin, insulin, and bovine serum albumin as protein components. By using the cell lines derived from RAG2(−/−) mice, which do not differentiate into c-*kit*$^-$ stage, we have evaluated the role of each protein in the cell cycle progression of c-*kit*$^+$IL-7R$^+$ B precursor cells. Since B precursor cells can grow without insulin, c-*kit* remains a sole functional receptor tyrosine kinase for their growth. While both c-*kit* ligand (KL) and IL-7 are the requisite molecules for sustained proliferation of B precursor cells, each molecule plays distinct roles. IL-7 starvation results in prompt arrest of the cells at G1. An accumulation of the cells in the mitotic phase was also detected. Thus, the major role of IL-7 is to regulate the G1/S transition and the process of cytokinesis of B precursor cells. Although prolonged KL starvation over 48 h resulted in accumulation of G1 cells, its effect could not be detected within 24 h, which is long enough for all the cells to complete one cell cycle. This suggests that KL might be involved in the cell cycle progression of B precursor cells in a manner that its signal could still be effective in the one or two cell cycles that follow. Although molecular nature of the signals underlying the present observation awaits future investigation, the method described in this report would provide a useful model system for investigating the signaling pathways that are involved in the cell cycle progression of B precursor cells.

B cell genesis in adult mouse is regulated by a meshwork of stromal cells in the bone marrow (1, 2). Discovery of a culture condition that supports sustained B cell genesis in vitro (3, 4) and subsequent establishment of B lymphopoiesis-supporting stromal cell lines (5–8) remarkably boosted our understanding of molecular and cellular basis of intramarrow B cell genesis of the mouse. On the basis of this progress, a number of stromal cell-dependent culture systems are now available for manipulating the proliferation of B precursor cells in vitro, thereby enabling us to measure the frequency of clonogenic B precursor cells at nearly 100% efficiency, establish primary culture of B precursor cell from various sources, and propagate them up to the numbers sufficient for biochemical analysis (9–12). Among a variety of molecules that have been identified to be expressed in the B lymphopoiesis-supporting stromal cell lines, IL-7 (13–15), the c-*kit* ligand (KL)[1] (16–18), and insulin-like growth factors (19) have been reported to stimulate the proliferation of B precursor cells and are available in the recombinant form. With this situation in mind, the present study aims to determine the minimum signals required for the cell cycle progression of a subset of normal B precursor cells that expresses both c-*kit* and IL-7 receptor (IL-7R). Our results clearly demonstrated that sustained proliferation of B precursor cells did occur under a fully defined culture condition including IL-7, KL, transferrin, insulin, and BSA as protein components. The proliferation of thus established B precursor cell lines requires both IL-7 and KL, while each molecule plays a distinctive role in the cell cycle control. We believe that the primary culture of B precursor cells described in this study will provide a unique model system where two distinct classes of receptors, namely cytokine receptor and receptor tyrosine kinase collaborate for cell cycle progression.

## Materials and Methods

*Animals.* C57BL/6(B6) and BDF1 mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The RAG2 gene knockout

[1] *Abbreviations used in this paper:* IL-7R, IL-7 receptor; KL, c-*kit* ligand; mSFO2, serum-free medium modified from SFO2 medium.


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(RAG2(−/−)) mouse strain was a kind gift from Dr. F. W. Alt (Howard Hughes Medical Institute, The Children's Hospital, Boston, MA) (20) and was maintained in our animal colony.

*mAbs and Cell Staining.* Anti-B220(RA3-6B2) (21), anti-c-*kit* (ACK2 and ACK4) (22, 23), and anti-IL-7R (A7R34) (24) mAbs were purified from ascites fluid as described previously (10, 15). Fluorescent isothiocyanate (Pierce Chemical Co., Rockford, IL) or *N*-hydroxysulfosuccinimide–biotin (Pierce) was conjugated to mAbs and used for staining. Phycoerythrin-conjugated avidine was obtained from Becton-Dickinson Laboratories (San Jose, CA). The stained cells were analyzed by Epics XL (Coulter Electronics Inc., Hialeah, FL) and sorted by FACS® Vantage (Becton-Dickinson).

*Cytokines and Cell Culture.* Murine recombinant IL-7 was prepared and titrated as described previously (10, 15). Recombinant KL was prepared as follows. cDNA corresponding to the extracellular domain (1–185 amino acids) of KL (25–27) was cloned by reverse transcription PCR (RT-PCR) and ligated to a yeast expression vector pYG10. This construct directs the recombinant KL to be secreted into medium. The yeast broth was concentrated by ultrafiltration and subjected to purification steps by MonoQ anion exchange chromatography (Pharmacia, Uppsala, Sweden) and subsequent phenyl-superose hydrophobic column chromatography (Pharmacia). The fraction containing active KL was identified by binding inhibition assay of anti-c-*kit* mAb ACK2 to c-*kit*$^+$IL-3–dependent mast cell lines. Maintenance of ST2 and PA6 stromal cell clones and limiting dilution analysis of clonogenic B precursor cells in serum-containing RPMI 1640 medium (GIBCO, Grand Island, NY) or a serum-free medium modified from SFO2 medium (mSFO2; Sanko Pure Chemical Co. Ltd., Chiba, Japan) were described in our previous papers (10, 15, 28). When B precursor cells were cultured without the help of stromal cell layer, mSFO2 supplemented with 1 mg/ml BSA (Fraction V; Sigma, St. Louis, MO), IL-7 (20 U/ml), and KL (50 ng/ml) was used throughout this study. In some experiments, crystallized BSA (Calbiochem, San Diego, CA) was used at the same concentration.

*Cell Cycle Analysis.* Amount of nuclear DNA by propidium iodide staining was carried out as follows. $10^6$ cells were incubated with 0.6% sodium citrate, 1 mg/ml RNase A (Takara, Shiga, Japan), 0.1% Triton X-100 (Wako Pure Chemical, Osaka, Japan), and 50 μg/ml propidium iodide (Sigma Immunochemicals, St. Louis, MO). The cells were washed with PBS containing 1% BSA and analyzed by EPICS XL. For BrdU labeling, cells were incubated with 5 μg/ml BrdU (Sigma). The harvested cells were fixed with 70% ethanol, treated with 2 N HCl/0.5% Triton X-100, and stained with FITC-conjugated anti-BrdU antibody (Progen Biotechnik GmbH, Heidelberg, Germany). After washing, the cells were resuspended with PBS containing 5 μg/ml propidium iodide and analyzed by EPICS XL. For calculating the proportions of each cell cycle stage, we used a gate that excluded apoptotic cells and doublet cells (doublet-excluding gate). This gate was set by measuring both peak and area fluorescence of propidium iodide, and only the cells that were plotted on the linear line were included in the assay. In one experiment, however, calculation was also made without setting this gate, because this doublet exclusion gate also excludes a proportion of the cells in the mitotic phase.

## Results

KL and IL-7 are sufficient for the long-term proliferation of B precursor cells in vitro. Previous studies of ours and other groups showed that IL-7 is an absolute requirement for B cell genesis both in vitro and in vivo (24, 29). Indeed, our recent studies demonstrated that IL-7R is expressed throughout the early phase of B lineage differentiation, before the expression of surface IgM (30). It was also demonstrated, however, that IL-7 alone is not sufficient for supporting sustained proliferation of B precursor cells (14, 15), indicating an involvement of other molecules for B cell genesis (10, 15). Among a number of molecules that are expressed in the stromal cell lines, KL might be a good candidate, because a fraction of B220$^{dull}$ cells express its receptor c-*kit* (22). Moreover, we have shown that anti-c-*kit* mAb suppressed both in vitro and in vivo B lymphopoiesis (16, 30). When we sorted B220$^+$c-*kit*$^+$ and B220$^+$c-*kit*$^-$ cells from fresh bone marrow and measured the frequencies of B precursor cells that are clonable on the PA6 layer in the presence of IL-7 (30), the frequencies of in vitro clonable B precursor cells were 5.3% and 0.06%, respectively, in B220$^+$c-*kit*$^+$ and B220$^+$c-*kit*$^-$ fractions. Thus, the next question addressed was whether these two signals are sufficient for the proliferation of c-*kit*$^+$IL-7R$^+$ B precursor cells. In fact, a recent study demonstrated that B precursor cell lines established on a B lymphopoiesis–supporting stromal cell clone ST2 could proliferate without the aid of stromal cell layer when both KL and IL-7 were present (31). Moreover, it was reported that sustained proliferation of normal B precursor cells is induced by recombinant IL-7 and KL (18). However, because of the presence of FCS in the culture media used in those studies, it has been difficult to formally exclude the possibility that additional growth factors are also required for the proliferation of B precursor cells. To determine whether IL-7 and KL are sufficient extracellular signals for the proliferation of c-*kit*$^+$IL-7R$^+$ B precursor cells, we have used a defined medium mSFO2 that can support the stromal cell-dependent B cell genesis in vitro (28). While mSFO2 alone can support the proliferation of B precursor cells on a stromal cell line ST2, our preliminary experiment indicated that 0.1% BSA is required for the growth factor–dependent proliferation of various hematopoietic cells in the absence of stromal cell layers. On this basis, we have attempted to establish primary cultures of B precursor cells with mSFO2 containing 0.1% BSA, 50 ng/ml KL, and 10 U/ml IL-7. Fig. 1 represents typical growth kinetics of bone marrow cells under our culture condition containing IL-7, KL, transferrin, insulin, and BSA as protein components. While initial decrease of the cell number and a lag period of cell growth during 2–5 wk did occur, sustained proliferation of the cells have been successfully induced in all experiments. Interestingly, no fibroblastic stromal cell layer was formed under this culture condition, even though macrophages generated in the culture formed an adherent layer. This is consistent with our previous observation that 0.1% BSA is toxic to the formation of stromal cell layer (28). Practically important is that B precursor cells proliferating in the Whitlock-Witte type culture could easily be transferred into this defined condition (data not shown). Hence, this culture condition could be used for establishing sublines from the B precursor cell lines that have been maintained on the stromal cells with a serum-containing medium.

*Change of Surface Phenotype during the Culture.* To characterize the processes taking place in this culture, we have analyzed the expression of c-*kit*, IL-7R, B220, and surface IgM

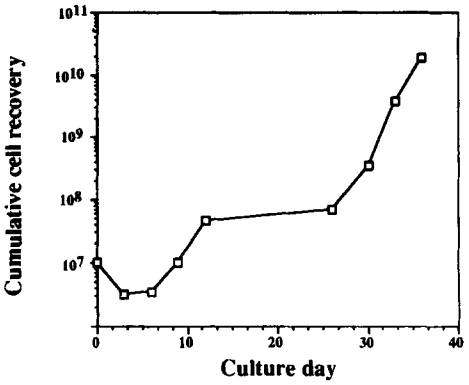


**Figure 1.** Cell growth during the establishment of the primary culture of B precursor cells. $10^7$ C57BL/6 bone marrow cells were cultured in mSFO2 medium containing IL-7, KL, transferrin, insulin, and BSA. Medium exchange was carried out twice a week. On the days indicated in this figure, the cells were harvested and reseeded into new dish to keep the cell concentration at $5 \times 10^5$/ml. At each cell harvest, the cumulative cell recovery was calculated.

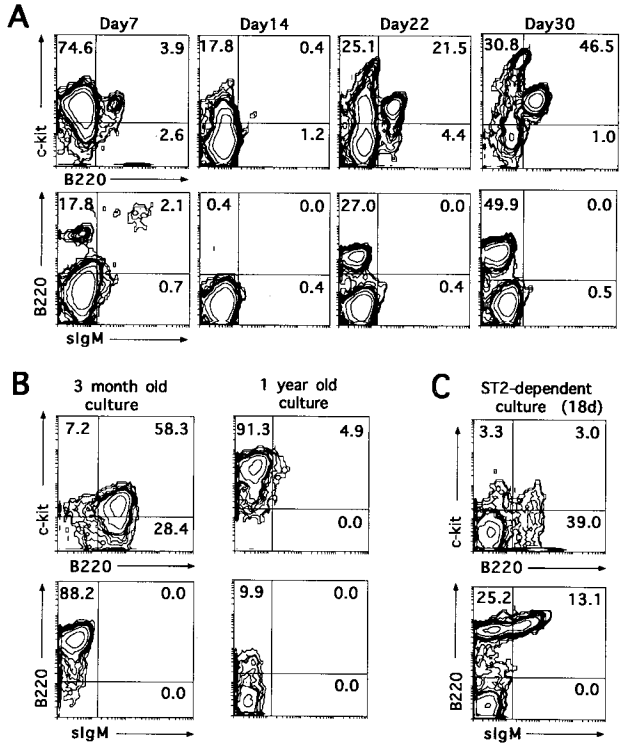


**Figure 2.** Surface phenotypes of the cells recovered from the primary culture of B precursor cells. (*A*) $10^7$ BDF1 bone marrow cells were cultured in mSFO2 medium containing IL-7, KL, transferrin, insulin, and BSA. At each medium exchange, an aliquot of cells were stained and analyzed for their surface phenotype. sIgM$^+$ cells have never been detected later than 14 d of the culture. (*B*) The surface phenotypes of the cells in 3-mo-old and 1-yr-old cultures. Upon longer term cultures, B220$^+$ cells eventually dominated the culture, but a considerable fraction of *c-kit*$^-$ cells were present in this culture, indicating that differentiation of the surface phenotype continued. RA3-6B2 epitope were often lost from the cells cultivated for >6 mo. Most cells presented in these figures were IL-7R$^+$ (data not shown). (*C*) $2 \times 10^6$ BDF1 cells were cultured on the ST2 cell layer under the Whitlock-Witte type condition in the presence of IL-7 (10 U/ml). At 18 d of culture, all the cells were harvested and analyzed for their surface phenotype. Many sIgM$^+$ cells were present in the culture.

(sIgM) at various phases of culture. As a control, we also analyzed the cells harvested from the culture of bone marrow cells on the ST2 stromal cell layer (Fig. 2). While mature B220$^+$sIgM$^+$ cells were detected during 10 d of culture, the proportion of B220$^+$ cells decreased to an undetectable level in the next two weeks (Fig. 2 *A*). This is caused by the proliferation of non-B lineage cells in response to KL. However, ~1 mo after the initiation of culture, the proportion of B220$^+$ cells in the cultured cells increased along with the decrease of non-B lineage cells. This decrease of non-B lineage cells correlates well with the loss of c-*kit* expression in the B220$^-$ population. While c-*kit*$^{hi}$ mast cells survived for long period of time, their growth rate declined 1 mo after the culture. Subsequently, only B220$^+$ cells remained as continuously proliferating cells under this culture condition, and they eventually become a sole cell population maintained under this culture condition. Thus initiated primary culture cells were able to be maintained for >1 y and to be kept frozen and thawed again at any phases of culture by the ordinary methods. However, such a long-term maintenance of B precursor cells as more than 6 mo usually resulted in loss of the epitope of B220 that is detected by RA3-6B2 mAb. Indeed, this is often the case of the long-term B cell culture on the stromal cell layer (our unpublished observation). Production of various subsets of B lineage cells were maintained more stably in the conventional bone marrow culture on the ST2 layer, indicating that the ST2 layer provides a microenvironment more preferable to B lineage cells. The most important difference between the conventional stromal cell-dependent culture and the culture under our defined condition is the ability to maintain sIgM$^+$ cells. Indeed, sIgM$^+$ cells never appeared in our defined culture condition, except during the first several days. This corroborates well with the previous observation that the transition from pre-B to B cells requires yet unknown additional stromal cell-derived molecules (7). All the cell lines thus far established except for those derived from the RAG2(−) mouse bore rearranged heavy chain gene organizations but did not differentiate into sIgM$^+$ cells even after transferring onto the ST2 layer that can support the entire process of B cell differentiation (data not shown). Thus, our culture condition selected the B precursor cells that failed to undergo productive heavy chain rearrangement.

*Minimum Growth Factor Requirement of B Precursor Cells.* Availability of primary culture of B precursor cells maintained under a fully defined culture condition now enables us to study the role of each protein in the cell cycle control of B precursor cells. One problem with using a freshly isolated growth factor–dependent cell line, however, is that they might differentiate as they proliferate in the culture, thereby giving rise to progenies that cannot survive in the same culture conditions because of changes in the growth signal requirements (Fig. 2 *B*). This problem was circumvented, though not completely, by using primary cultures established from RAG2(−/−) mice whose B cell differentiation is arrested at early stage (20). Indeed, most B220$^+$ cells in the bone marrow of the

RAG2(−/−) mouse are c-*kit*$^+$B220$^+$, while those in the bone marrow of the normal mouse are c-*kit*$^-$B220$^+$ (30). For this reason, several primary cultures of B precursor cells have been established from RAG2(−/−) mice in the same manner as shown in Fig. 1 and used in the subsequent experiments. Indeed, virtually all of the cells in the primary culture of RAG2(−/−) mice were IL-7R$^+$c-*kit*$^+$ (data not shown). First, to determine the minimum requirements for the growth of B precursor cells, we cultured them in the presence or absence of BSA, insulin, KL, and IL-7 (Fig. 3). Though BSA is an absolute requirement for the proliferation of B precursor cells, a highly pure crystallized BSA preparation has the same effect with BSA Fraction V. This indicates that the effect is not caused by the molecules contaminated in the BSA preparation. While increase of cell number was observed in the culture containing both KL and IL-7, starvation of either of the two molecules resulted in a cessation of cell growth. Interestingly, most cells died in 2 d by IL-7 starvation. On the other hand, decrease of cell number by KL starvation was more gradual than that by IL-7 starvation, though all the cultured cells eventually died within 10 d. This indicates that the sustained growth of the B precursor cells requires both IL-7 and KL, while the role of each factor in this process is different. Finally, the fact that B precursor cells could proliferate normally in the insulin-free medium indicates that c-*kit* remains a sole receptor tyrosine kinase involved in the proliferation of B precursor cells under this culture condition. While the preceding results indicate clearly that transferrin, BSA, IL-7, and KL are the minimum protein requirements for the proliferation of B precursor cells, it is important to note that all our attempts to clone the cells under this fully defined condition failed. In contrast, the same cells were able to be cloned on the ST2 layer with even BSA-free mSFO2 in the presence of IL-7 and KL at ~30% efficiency (data not shown). This indicates that additional molecules are indeed required for the proliferation of B precursor cells, particularly with respect to the proliferation of the single cells. Importantly, however, this stromal cell dependency disappears basically at high cell density of B precursor cells in the culture. This suggests that B precursor cells themselves are able to supply the signals that are sufficient for maintaining the cell cycle progression of B precursor cells in the presence of KL and IL-7. Hence, this density effect on cell growth might be similar to that defined by Gurdon et al. (32) as the community effect.

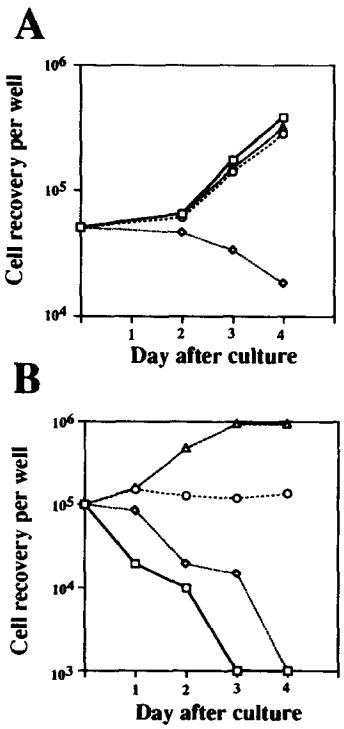


**Figure 3.** Growth requirements of the B precursor cells in the primary culture established from the bone marrow cells of RAG2(−/−) mouse. The primary culture of B precursor cells of a RAG2(−/−) mouse was established. 2 mo after the initiation of culture, the cells were harvested and used in the experiments. (*A*) 5 × $10^4$ cells in 100 μl mSFO2 were cultured in the 96-well plates. The control culture contained IL-7, KL, insulin, transferrin, and BSA. —□—, control; ····◇····, BSA (−); -○-, insulin (−); -△-, crystalized BSA. (*B*) $10^5$ cells in 100 μl mSFO2 were cultured in 96-well plates. KL and IL-7 were added in various combinations to this basal condition. Each value represents arythmetic mean of triplicate cultures. —□—, none; ····◇····, SCF; -○-, IL-7; -△-, SCF + IL-7.

*Role of IL-7 and KL in the Cell Cycle Progression of B Precursor Cells.* To identify at which stage of the cell cycle of B precursor cells IL-7 and KL play their roles, RAG2(−/−) B precursor cells that were maintained in the presence of both KL and IL-7 were starved from either IL-7 or KL for 24 or 48 h, and the DNA contents of the harvested cells were measured by propidium iodide staining (Fig. 4). Upon IL-7-starvation, rapid accumulation of the cells at G1 stage was induced. On the other hand, while accumulation of G1 stage was the eventual outcome of KL starvation, this occurred gradually. This result indicates that IL-7 plays a crucial role in the G1/S progression of B precursor cells. Though prolonged KL starvation also resulted in the G1 arrest of B precursor cells, it is not clear if KL directly regulates the G1/S transition. The fact that virtually all cells were labeled with BrdU by 24 h incubation (data not shown) indicates that all B precursor cells can complete at least one cycle within 24 h. If KL is a crucial signal for G1/S transition, the effect of its starvation should be seen earlier. In the course of this study, however, we found that the doublet-excluding gate used for the previous analysis seemed to also exclude a proportion of cells that are in the mitotic phase, probably because of their complicated shape. This was noted by a finding that the cells in the IL-7–starved culture contained a significant number of mitotic cells. This suggests that IL-7 may play some roles in the progression of the cytokinesis. If this is the case, however, these mitotic cells should be detected in the G2/M area. To resolve this problem, we carried out a two-parameter cell cycle analysis in which BrdU labeling and propidium iodide staining are combined and compared the staining patterns before and after setting the doublet-excluding gate. RAG2 (−/−) B precursor cells were incubated under either IL-7 or KL starvation for 22 h, and the cells were labeled by BrdU

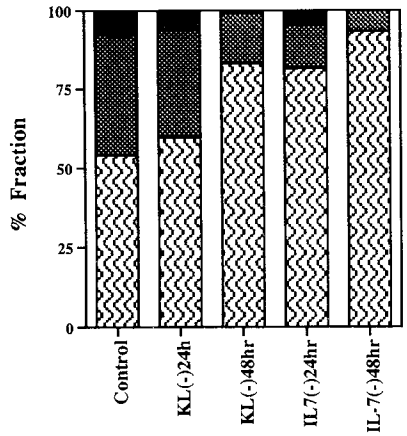


**Figure 4.** Cell cycle analysis of B precursor cells cultured in the absence of either IL-7 or KL. The cells in the 4-mo-old primary cultures of B precursor cells from a RAG2(−/−) mouse were harvested, washed, and cultured in the absence of either IL-7 or KL at a concentration of 5 × $10^5$ cells per ml. The cells were harvested 24 or 48 h after the culture, and the proportions of each cell cycle stages were calculated after propidium-iodide staining. The analysis was carried out with the doublet-excluding gate. Each column represents the arithmetic mean of duplicate cultures. ▨, G1; ▩, S; ■, G2/M.

during the last 30 min of the culture. The cells were harvested and stained with FITC-BrdU and propidium iodide and analyzed with a flow cytometer with or without setting the doublet excluding gate (Fig. 5). The staining pattern of the gated population corroborated well with the results in Fig. 4 that IL-7 starvation resulted in the G1 arrest of the cells, while short-term starvation of KL had no significant effect on the cell cycle progression. Moreover, two-parameter analysis provided clearer demonstration of the decrease of S phase cells upon IL-7 starvation. Interestingly, when the same samples were analyzed but without setting the gate, however, it was demonstrated that KL starvation resulted in a significant increase of apoptotic cells. More interesting is that IL-7 starvation induced G2/M as well as G1 arrest of the cell cycle. A part of this increase of the cells in G2/M phase might be a result of counting the actual doublet cells. However, the doublet cells alone may not be able to account for such a big increase of the proportion of G2/M fraction as 4.7% for the gated and 16.4% for the ungated populations. In fact, no such increase was observed in the cultures with IL-7. Because histological examination demonstrated an increase of the proportion of mitotic cells upon IL-7 starvation, those cells could be the ones responsible for this increase of G2/M fraction. These results suggest that IL-7 may play a role also in the cytokinesis of B precursor cells.

## Discussion

*Defined Culture Condition Supporting Sustained Proliferation of B Precursor Cells.* An important goal for the investigation of growth control of any cell types is to establish the defined conditions under which the proliferation of the freshly isolated cells are able to be supported in vitro. However, because of the complexity of control mechanisms that regulate proliferation, survival, and death of any given cell types, this goal has not yet been attained for most of the cells that make up a mammalian body. In contrast, we here reported that B precursor cells can be listed as a cell type that is able to be maintained in vitro under a fully defined condition. The

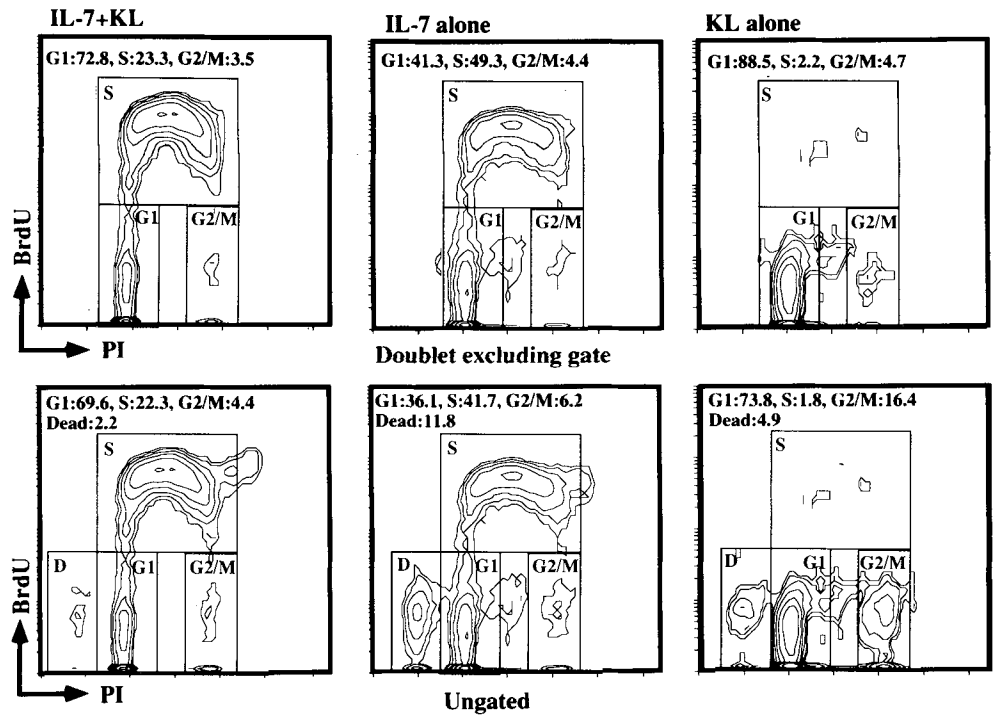


**Figure 5.** Effect of IL-7 or KL starvation on DNA synthesis of B precursor cells. (*A*) The B precursor cells in the culture of RAG2(−/−) mouse were incubated for 22 h in the presence or absence of KL or IL-7. The cells were incubated with 5 μg/ml BrdU for 30 min before harvest. The harvested cells were analyzed by two-color flow cytometry after staining with FITC-anti-BrdU mAb and propidium-iodide (*PI*) with (*upper panels*) or without (*lower panels*) setting the doublet-excluding gate. Though the proportion of G2/M phase in the IL-7-containing cultures did not change with or without the doublet-excluding gate, its proportion in the IL-7-starved culture changed from 4.7 to 16.4% after removing the doublet-excluding gate.

defined medium used in this study is a nearly protein-free medium mSFO2 described in our previous paper (28), and it contains only 10 μg/ml protein consisting of insulin and transferrin. To this, we have added 1 mg/ml BSA, 50 ng/ml KL, and 10 U/ml IL-7. Under these conditions, the primary culture of B precursor cells is easily established without help of B lymphopoiesis-supporting stromal cell lines that have been used for this purpose (5–8). Since insulin is not required for the proliferation of thus established B precursor cell lines, the cell cycle progression of B precursor cells should be a consequence of the activations of two signaling pathways initiated by IL-7R, a cytokine receptor family molecule, and c-*kit*, a receptor tyrosine kinase. It is noteworthy, however, that a community effect does exist for B precursor cells, since single cells cannot proliferate under our culture condition, while the same cells proliferated at higher density. Clearly, this community effect is not caused by the contamination of non-B lineage cells because our defined culture condition does not allow the proliferation of stromal cell components (28), and we have obtained the same result by using sorted pure c-*kit*$^+$ IL-7R$^+$ cell population (our unpublished observation). Thus, at present, the minimum signals required for the cell cycle progression of B precursor cells detectable under our culture condition could be formulated as IL-7, KL, and the community effect whose molecular basis is yet to be determined. This conclusion is an extension from the previous studies of ours and other groups showing that a subset of intramarrow B precursor cells could proliferate in vitro in response to IL-7 and KL (16–18, 31). However, as single B precursor cells could not proliferate under our culture condition, it is difficult to determine how much proportion of c-*kit*$^+$IL-7R$^+$ B precursor cells are able to proliferate under our culture condition. Indeed, Faust et al. reported the existence of a different subset of B precursor cells that did not react to IL-7 nor KL despite of their expression of IL-7R and c-*kit* (33). It is interesting to know which defined culture conditions are able to support the proliferation of the subset described by Faust et al. (33). Nevertheless, the present study does not exclude the involvement of other molecules like insulin like growth factor (19), IL-11 (18), or other stromal cell molecules (34–37) in the cell cycle progression of c-*kit*$^+$IL-7R$^+$ B precursor. On the contrary, we believe that the actual intramarrow process regulating the proliferation of B precursor might be a much more complex process involving multiple factors because the level of cell growth under our culture condition was slower than that which is able to be induced with the stromal cell layer or in the presence of FCS. Indeed, we detected a clear difference in the ability to maintain sIgM$^+$ cell production between this defined culture condition and the ST2-dependent culture, though sIgM$^-$ B lineage cells eventually dominated, even in the stromal cell- dependent culture (4, 9). Thus, as is the case for any type of tissue culture, the present culture system provides a highly selective condition under which only a certain subset of B precursor cells are maintained. If only a minimum level of the proliferation and differentiation of B precursor cells are supported under our culture condition, this culture condition would be useful for evaluating the activity of those additional molecules. At any rate, the culture condition described here is the first defined condition that is able to support the sustained growth of B precursor cells in the absence of stromal cell lines.

*Role of IL-7 and KL in the Cell Cycle Progression of B Precursor Cells.* While the molecular nature of the community effect or cell density effect remains to be determined, IL-7R and c-*kit* eventually remain as minimum receptors for extracellular growth factors required for the cell cycle progression of B precursor cells. In this study, we used the culture of RAG2(−/−) mice since our previous study demonstrated that B lineage differentiation of this strain is arrested at the IL-7R$^+$c-*kit*$^+$ stage (30). Thus, basically no differentiation into c-*kit*$^-$ stage is expected to occur in this culture, thereby enabling us to disregard the possibility that inhibition of the cell proliferation is induced by growth receptor downregulation. We have also confirmed that removal of one cytokine did not affect the expression level of the receptor for the other cytokine (data not shown). Hence, two receptors are always available on the surface of B precursors in our experimental condition. While both KL and IL-7 are required for the sustained cell cycle progression of B precursor cells, each signal seems to play distinct roles. The effect of IL-7 starvation is characterized as (*a*) rapid decrease of viable cells, (*b*) accumulation of G1 phase, and (*c*) accumulation of the mitotic cells in the G2/M phase. These observations indicate that the progression of G1/S transition and cytokinesis of B precursor cells is dependent on IL-7. Prompt cell death upon IL-7 starvation may also suggest that IL-7 functions as a survival factor. However, it is usually difficult to separate the cell survival activity from the cell cycle progression activity of a given molecule when it is essential for the cell cycle progression of such cells since those that need to be kept cycling or otherwise die. If the survival factor should be able to maintain the survival of the resting cells, IL-7 could not be such for B precursor cells, as rapid cell death of B precursor cells was induced by G1 blockers such as thymidine even in the presence of IL-7 and KL (our unpublished observation). Moreover, the recent result of Griffiths et al. (38) showed that presence of IL-7 could not rescue the cell death of an IL-7–dependent cell line induced by corticosteroid. Thus, rapid cell death upon IL-7 starvation could simply be an outcome of cell cycle arrest and does not necessarily indicate that IL-7 is a survival factor. It is of note that the number of cells in the culture remained constant upon KL starvation, although cell cycle progression continued. This indicates that a considerable fraction of cells may also die by KL starvation, even in the presence of IL-7. Indeed, the result in Fig. 5 demonstrated an increase of apoptotic cells by KL starvation. Thus, both molecules, when assessed in terms of cell death upon starvation, might likely be classified as cell survival factors. Taken together, whether or not IL-7 is a survival factor for the B precursor cells is yet to be elucidated, while it is clear that IL-7 directly regulates the G1/S transition and cytokinesis of the B precursor cells. The present study failed to specify the role of KL in the cell cycle progression of B precursor cells, although it is required for their sustained proliferation. Upon

KL starvation, cell cycle progression of B precursor cells seems to continue because no specific loss of each cell cycle stage was detected during 24 h. In contrast, prolonged starvation did arrest the cells at the G1 stage. Considering the fact that the doubling time of the cells used in this study is <24 h, KL could not be the signal that directly regulates the G1/S transition. It could be that KL plays a similar role as TCR stimulation in IL-2–dependent T cell proliferation. Previous studies have shown that TCR stimulation induces a series of events that bring the resting T cells into G1 phase and make them able to proliferate in response to IL-2 by up-regulating IL-2 receptor expression (39, 40). With regard to B precursor cells, however, rapid cell death occurs upon keeping them at the resting phase so that basically no resting stage exists. Moreover, KL stimulation has no effect on the expression level of IL-7R. Hence, the function of KL for B precursor cells might be different from that of TCR stimulation for the resting T cells. The second possibility is that KL is actually a survival factor for B precursor cells. In fact, in the previous studies of our group and other groups on the in vitro proliferation of primordial germ cells, KL was suggested to be a survival factor rather than the cell cycle progression factor (41–43). The results in Fig. 5 also demonstrate that the proportion of apoptotic cells did increase by KL starvation. Accepting this notion, survival activity of KL appeared to be effective only on the cycling cells because KL could not rescue the cell death of G1 arrested B precursors induced by IL-7 starvation. Though it is plausible that cycling cells also require a survival signal, this assumption is not sufficient to explain the reason why the cells in the G1 phase accumulated upon long-term KL starvation. At the present time, we favor the third possibility that while KL is the requisite signal for cell cycle progression of B precursor cells, the KL-induced signal could be maintained at the level sufficient for functioning in one or two cell cycles that follow starvation. Nevertheless, to determine which of those possibilities is indeed the case, we need to understand the molecules that transmit the signals generated at IL-7R and c-*kit* to the molecules directly regulating the sequential events during the G1/S transition of B precursor cells. To this end, B precursor cells cycling under a fully defined culture condition and mAbs against c-*kit* and IL-7R (22–24) will provide important tools. Finally, we present in this report an oversimplified model system to deal with the cell cycle progression of normal c-*kit*$^+$IL-7R$^+$ B precursor cells. Indeed, this model system is consisted of B precursor cells whose differentiation is blocked at c-*kit*$^+$IL-7R$^+$ stage, thereby avoiding the spontaneous change of the growth signal requirement of the target cells. Obviously, the behavior of the cells described here might be different from those in the actual bone marrow, where multiple factors are involved in different combinations, and proliferation, differentiation, and cell death are occurring at the same time. However, it might be clear that the population dynamics of the intramarrow B lymphopoiesis, because of its enormous complexity, can not be elucidated without understanding the principal behavior of each distinct stage of B lineage cells in response to different signals. In this context, we believe that this study provides starting material that will be continuously modified to create a newly defined condition that more closely resembles actual bone marrow.

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