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Identification of The Target Genes Critical for Hematopoietic Cell Cycle Induced by IL-2 and Other Cytokine Receptors

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CONTENTS

SUMMARY

Hematopoietic cytokines are critical inducers of proliferation and differentiation of hematopoietic cells, but the mechanisms of cytokine-induced signal transduction are not well understood. The cell cycle progression of the hematopoietic cell line, BAF-B03 is induced by IL-2, IL-3 and EPO, but not by EGF, when the cells express the respective receptors. As an approach to elucidate the mechanisms underlying cytokine-induced signaling, I examined expression of the target genes induced by these cytokines in BAF-B03 cells. The target genes include nuclear proto-oncogenes such as c-*jun*, c-*fos* and c-*myc*. These genes were induced by IL-2, IL-3 or EPO in this cell line. Intriguingly, the studies of BAF-B03-derived cells expressing mutant cDNAs for the human IL-2 receptor β chain (IL-2Rβ) revealed a bifurcation of the IL-2 signaling pathways. One pathway leads to the induction of the c-*jun* and c-*fos* genes, presumably mediated by a *src*-family protein tyrosine kinase(s), and another pathway may be linked to the induction of the c-*myc* gene. EGF induced-expression of the c-*myc* gene in BAF-B03 cells was impaired, and the cells failed to proliferate in response to EGF. However, ectopic expression of an exogenous c-*myc* gene restored their ability to proliferate in response to EGF, suggesting that c-*myc* plays a critical role in the hematopoietic cell cycle.

INTRODUCTION

The hematopoietic system consists of highly specialized types of cells which were differentiated from a pluripotent stem cell (Spangrude et al., 1988). The proliferation and differentiation of these cells are tightly regulated by a number of hematopoietic cytokines, including interleukins and colony stimulating factors (Metcalf, 1989). Cytokines transduce their signals through the interaction with respective membrane receptors, and elicit a series of biochemical events which lead to the expression of genes responsible for their effects.

Cytokine Receptor Superfamily

Recent advances of isolation of genes encoding hematopoietic-cytokine receptors have led to the identification of a new cytokine receptor superfamily (Figure 1) (D'Andrea et al., 1989b; Bazan, 1990; Cosman et al., 1990; Hatakeyama, 1993; Miyajima et al., 1992). The members of this receptor superfamily have the common motifs, that are a set of four conserved cystein residues and a segment consisting of Trp-Ser-X-Trp-Ser (WS motif), in the extracellular domains. In contrast, the cytoplasmic domains of each member are rather diverged and don't possess common structural motifs. Furthermore, apparent catalytic domains, such as a tyrosine kinase domain that was found in the epidermal growth factor receptor (EGFR) (Ullrich and Schlessinger, 1990) or the colony stimulating factor-1 receptor (CSF-1R) (Sherr, 1991), were not observed (Figure 1). Indeed, little is known about the machineries of intracellular signaling mediated by these hematopoietic-cytokine receptors.

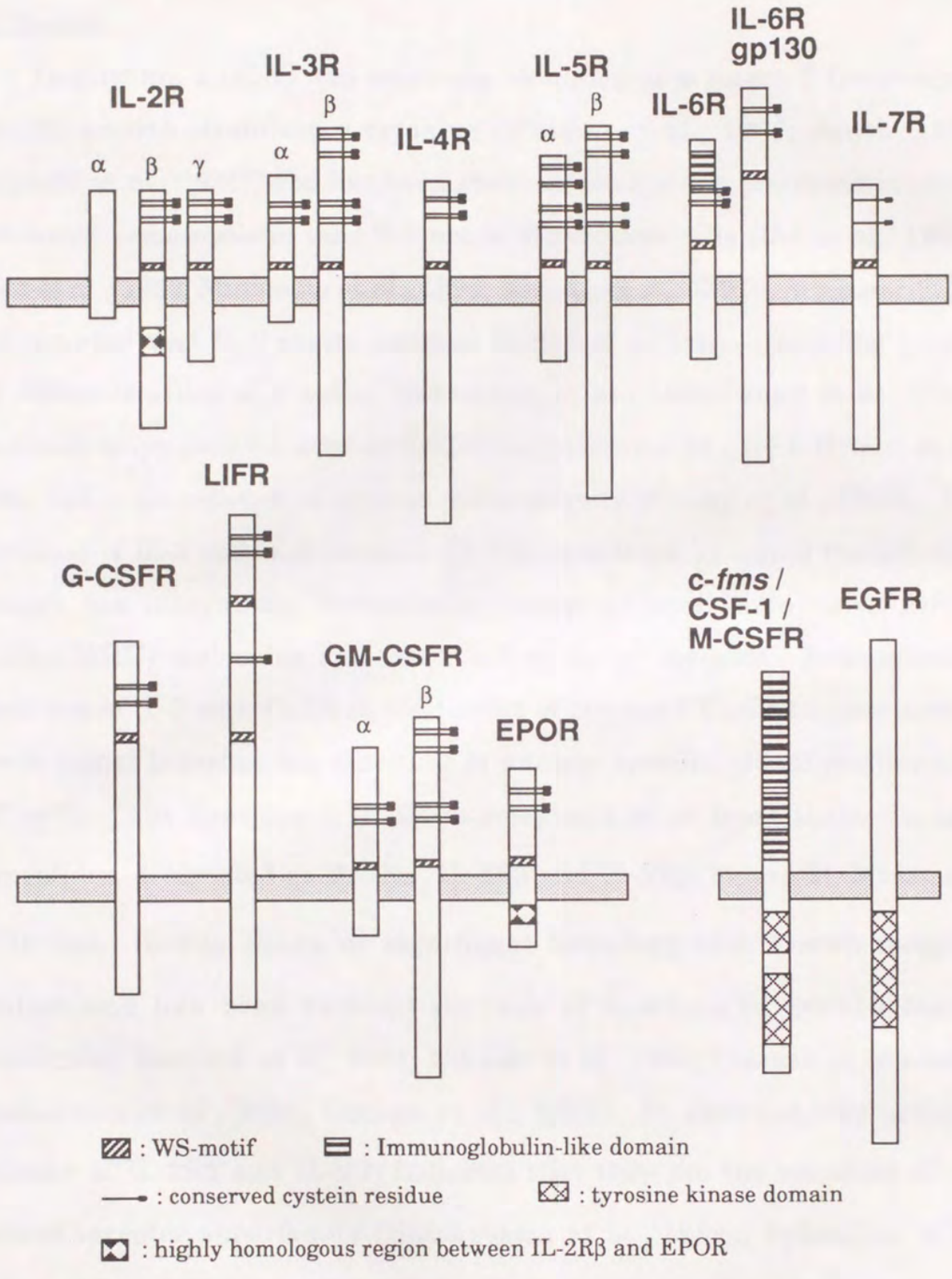


Figure 1. Structure of The Cytokine Receptors.

Abbreviations: IL, interleukin; CSF, colony stimulating factor; G-CSF, granulocyte-CSF; LIF, leukemia inhibitory factor; GM-CSF, granulocyte-macrophage-CSF; EPO, erythropoietin; M-CSF, macrophage-CSF; EGF, epidermal growth factor.

IL-2 System

Interleukin 2 (IL-2) was originally identified as a potent T lymphocytes (T cells) growth-stimulatory cytokine (Morgan et al., 1976; Smith, 1988; Taniguchi et al., 1986), and has been shown to mediates a proliferative signal in cultured hematopoietic cells but not in fibroblastic cells (Doi et al., 1989a; Tsudo et al., 1989; Minamoto et al., 1990; Tanaka et al., 1991). Moreover, it has been reported that IL-2 exerts multiple biological activities, including growth and differentiation of B cells (Waldmann et al., 1984; Jung et al. 1984), generation of lymphokine-activated killer cells (Lotze et al., 1981; Grimm et al., 1982), and augmentation of natural killer activity (Henny et al., 1981). The expression of IL-2 and IL-2 receptor (IL-2R) is induced by signal transduction through the interaction between an antigen/major histocompatibility complex(MHC) molecules and the T cell receptor complex. Subsequently interaction of IL-2 with IL-2R on the surface of activated T cells triggers a set of growth signal transduction resulting in antigen specific, clonal proliferation of T cells. The functional IL-2R is composed of at least three distinct polypeptides, designated as IL-2Rα, IL-2Rβ and IL-2Rγ (Figure 2) (Minami et al., 1993a). IL-2Rα shows no significant homology with known receptor families and has been verified its lack of function in growth signal transduction (Leonard et al., 1984; Nikaido et al., 1984; Cosman et al., 1984; Hatakeyama et al., 1985; Greene et al., 1985). In contrast, the primary structure of IL-2Rβ and IL-2Rγ indicates that they are the members of the cytokine receptor superfamily (Hatakeyama et al., 1989a; Takeshita et al., 1992). Although the role of IL-2Rγ in IL-2 signaling remains obscure, the evidences has accumulated that IL-2Rβ plays a central role in IL-2-induced signal transduction. Hatakeyama et al. have reported that the transformant expressing the human IL-2Rβ by cDNA transfection in an IL-3-dependent

murine hematopoietic cell line, BAF-B03, which express endogenous mouse IL-2Rα and IL-2Rγ, can become capable to proliferate in response to IL-2 (Hatakeyama et al., 1989b). Furthermore, the cytoplasmic region of IL-2Rβ which is critical for signal transduction has also been identified by expressing IL-2Rβ mutant cDNAs in BAF-B03 cells. The previous results suggested that the "serine rich"region of IL-2Rβ plays a critical role for IL-2-induced proliferative signals in this cell line (Figure 2) (Hatakeyama et al., 1989b; Mori et al., 1991). It is worth noting that this "serine rich" region shows high degree of homology (-35%) with the corresponding region of the erythropoietin receptor (EPOR) (Figure 1) which is also the member of cytokine receptor superfamily (D'Andrea et al., 1989a; D'Andrea et al., 1989b), suggesting that the existence of a common signaling pathway in part between them. On the other hand, it has been reported that the "acidic" region of IL-2Rβ is primarily responsible for associating a lymphocyte-specific non-receptor tyrosine kinase, p56^{lck}, when examined in COS cells by transient cDNA expression experiment (Hatakeyama et al., 1991). Protein tyrosine kinases have been recognized as important transducers of proliferative signals (Hunter and Cooper, 1985). Following IL-2 stimulation of BAF-B03 cells expressing IL-2Rβ and of the IL-2-dependent T cells, a rapid increase in tyrosine phosphorylation of cellular substrates was observed, suggesting that the tyrosine phosphorylation events upon IL-2 stimulation may transduce some signals in this cells (Horak et al., 1991; Satoh et al., 1992; Minami et al., 1993b). In BAF-B03 cells which do not express p56^{lck}, another *src*-family kinase, p59^{fyn}, interacts with IL-2Rβ, and can account for the IL-2-induced tyrosine phosphorylation events in the cells (Kobayashi et al., 1993). However, the nature of this signaling pathway still remains obscure.

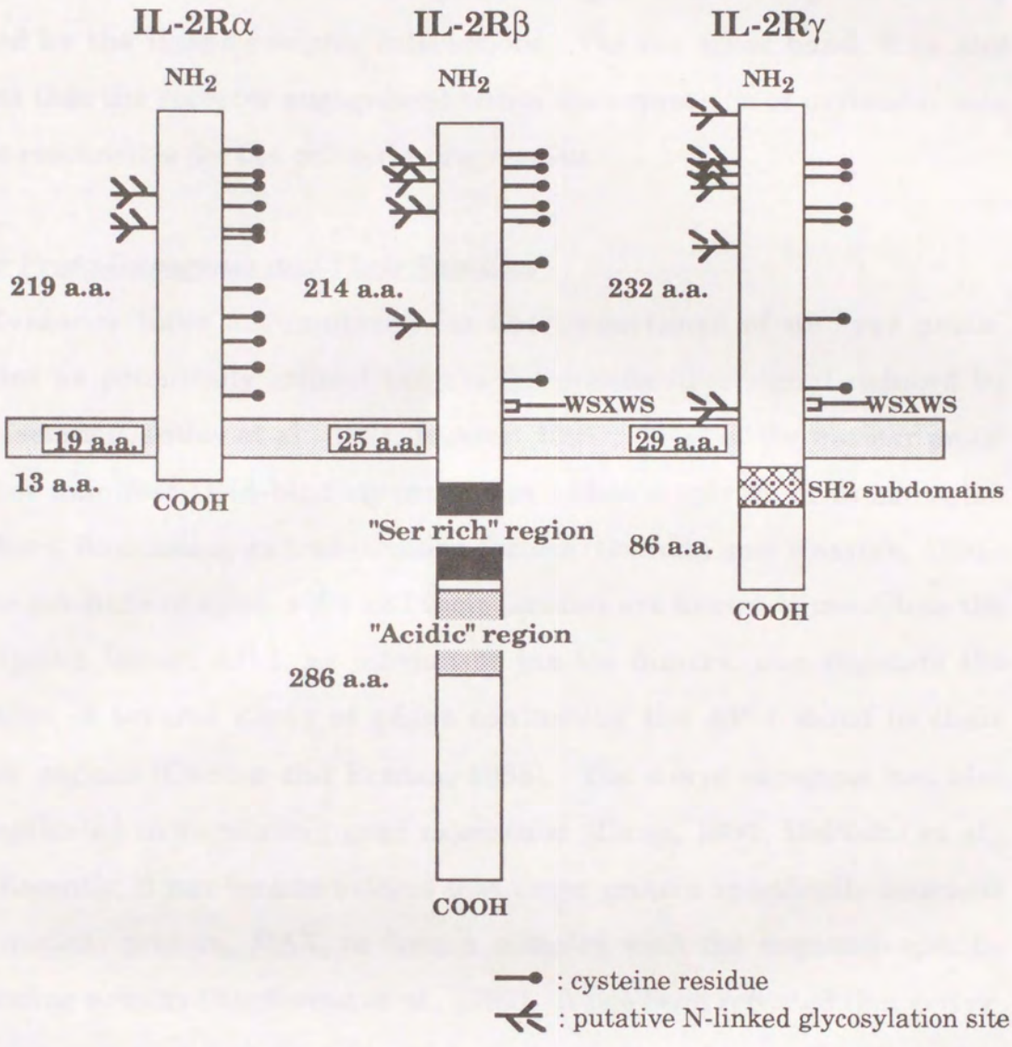


Figure 2. The Three Components of IL-2R.

In order to understand the mechanisms of cytokine receptor-mediated proliferative signal transduction, it is reasonable to analyse signal transducers interacting with the cytoplasmic region of the receptors or being activated by the ligand-receptor interactions. On the other hand, it is also apparent that the receptor engagement elicits the expression of particular sets of genes responsible for the cell cycle progression.

Nuclear Proto-Oncogenes and Their Families

Evidence have accumulated for the importance of nuclear proto-oncogenes as potentially critical targets for proliferative signal induced by growth factors (Cantley et al., 1991; Hunter, 1991). Most of the nuclear proto-oncogenes manifest DNA-binding properties either singly or in combination with others, functioning as transcription factors (Gutman and Wasylyk, 1991). The gene products of c-*jun*, c-*fos* and their families are known to constitute the transcription factor, AP-1, as jun-jun or jun-fos dimers, and regulate the expression of several kinds of genes containing the AP-1 motif in their promoter regions (Curran and Franza, 1988). The c-*myc* oncogene has also been implicated in regulating gene expression (Dang, 1991; DePinho et al., 1991). Recently, it has become evident that c-*myc* protein specifically interacts with a nuclear protein, MAX, to form a complex with the sequence-specific DNA-binding activity (Blackwood et al., 1992). It has been reported that c-*myc* gene expression may link to many biological events such as cell cycle entry, DNA replication, differentiation, apoptosis (Dang, 1991; DePinho et al., 1991), however, the function and target genes of c-*myc* protein in cell cycle progression are not fully understood.

Cell Cycle-Related Genes

The expression of nuclear proto-oncogenes are followed and accompanied by progression through cell cycle, wherein the expression of cell

cycle regulators such as cyclins and cyclin dependent kinases (cdks) were observed (Hunter and Pines, 1991). Cyclins are the regulatory subunits of cdks that govern both G1/ S and G2/M transitions, and regulate the activation of cdks by their periodic expression. In mammalian cells, the best characterized cyclin is cyclin B, which can form complex with cdc2 kinase to regulate both mitotic entry and exit (Murray and Kirschner, 1989; Nurse, 1990). Cyclin A can associate with cdc2 and with a related kinase cdk2 during S and G2 phase of cell cycle (Pines and Hunter, 1990; Ninomiya-Tsuji et al., 1991; Elledge and Spottswood, 1991; Tsai et al., 1991; Pagano et al., 1992; Rosenblatts et al., 1992; Elledge et al., 1992), although the function of cyclin A is still unclear. Cyclins C, D1, D2, D3 and E have been identified as G1 cyclins, which involved in G1/S transition (Lew et al., 1991; Motokura et al., 1991; Xiong et al., 1991; Matsushime et al., 1991; Koff et al., 1991). Although these genes appear to be functionally redundant, the recent observations indicate that the functions of these cyclins are regulated by the cell-type specific expression or their catalytic partners. For example, cyclins D1 and D3, whose catalytic partner are related kinases, cdk4 and cdk5, were identified as G1 cyclins specifically expressed in CSF-1-dependent macrophages and IL-2-dependent T cells, respectively (Matsushime et al., 1991; Matsushime et al., 1992). By contrast, the partner of cyclin E is known to be the cdk2 (Koff et al., 1991).

As an approach to gain further insights into growth signal transduction mediated by the cytokine receptors, I tried to examine expression of several target genes induced upon cytokine stimulation of the hematopoietic cells (Shibuya et al., 1992). Although BAF-B03 cells were originally selected for their IL-3 dependency, they can also proliferate in response to other cytokines, such as IL-2 and EPO, when they express their respective receptors by cDNA transfection. On the other hand, EGFR, that is a receptor tyrosine kinase, fail to stimulate the proliferation of the BAF-B03 cell in the presence of EGF. In

this study, I analysed the expression of nuclear proto-oncogenes, cyclins and cdks upon stimulation of BAF-B03-derived cells with several growth factors, including IL-2, IL-3, EPO and EGF. The experimental results suggest that the growth signal transduction induced by IL-2, IL-3 or EPO-stimulation may be very similar in respect of the expression of several genes in the cells. On the other hand, in the case of IL-2R, the data has revealed the presence of at least two different signaling pathways for proto-oncogene induction. One pathway may be linked to protein tyrosine kinase activation and leads to the induction of c-*fos*, c-*jun*, and related genes, and another one leads to the induction of c-*myc* gene by an as yet unknown mechanism. Although EGF cannot stimulate both the induction of c-*myc* and cell proliferation, the human c-*myc* expressed by DNA transfection can render the cells to proliferate in response to EGF, implying that c-*myc* plays a critical role for cell cycle progression in BAF-B03 cells.

RESULTS

Progression of BAF-B03 Cell Cycle by Cytokines

In order to identify target genes induced by several cytokines in hematopoietic cells, I used the following BAF-B03-derived cell lines. The hematopoietic cell line BAF-B03 is a subclone of the IL-3-dependent murine pro-B cell line BA/F3 (Palacios and Steinmetz, 1985; Collins et al., 1988; Daley and Baltimore, 1988). This cell line expresses the endogenous mouse IL-2Rα and γ, exhibiting a nonfunctional low-affinity receptor for IL-2. F7 cells expressing the human IL-2Rβ by a cDNA transfection display both low and functional high-affinity forms of IL-2R, and can proliferate in medium containing human recombinant IL-2 (Hatakeyama et al., 1989b). This observations suggest that IL-2Rβ has a critical role in transducing the IL-2-induced proliferative signals in hematopoietic cells. A15 cells are also the BAF-B03-derived transformants which express the human IL-2Rβ mutant lacking the cytoplasmic "acidic" region (a.a. 312 to 383) of IL-2Rβ. It has been reported that a *src*-family protein tyrosine kinase (PTK) can physically associate with the "acidic" region (Hatakeyama et al., 1991; Minami et al., 1993b; Kobayashi et al., 1993). In fact, IL-2Rβ lacking this "acidic" region fails to induce tyrosine phosphorylation upon IL-2 stimulation (Satoh et al., 1992; Minami et al., 1993b). Nevertheless, A15 cells can proliferate in response to IL-2, suggesting that the association of IL-2Rβ with *src*-family kinase is not necessary for the proliferation of the BAF-B03 cells. F7E2 cells are F7-derived cells expressing both IL-2R and EPOR, obtained by transfecting mouse EPOR cDNA into F7 cells, can proliferate in the response to EPO as well as IL-2 (Hatakeyama et al., 1992). Furthermore, other groups have shown that the

BAF-B03 cells can proliferate in response to other hematopoietic cytokines such as IL-6 and granulocyte-CSF (G-CSF) (Hibi et al., 1990; Fukunaga et al., 1991), when the cells express the respective receptors by cDNA transfection, implying the presence of an identical or closely-related mechanisms for growth signal transduction induced by these cytokines. In addition, I generated an EGFR expressing clone, BER2, by transfecting the human EGFR cDNA into the BAF-B03 cells. EGFR, which is a receptor-type protein tyrosine kinase, is known to stimulate proliferation of the epidermal, epithelial, and fibroblastic cells (Ullrich and Schlessinger, 1990). Meanwhile, it has been reported that the EGFR could not induce cell proliferation of the bone marrow-derived cells, BA/F3 cells, and another IL-3-dependent cell line, IC2 (Rüden and Wagner, 1988; Wang et al., 1989). In the case of BER2 cells, although rapid autophosphorylation of EGFR on tyrosine residues was observed upon EGF-stimulation (data not shown), EGF could not induce the proliferation of BER2 cells.

These four BAF-B03-derived transfectants were subjected to cell cycle analysis by flow cytometry (Figure 3). When these cells were cultured for 15 hrs in the absence of cytokines, about 80% of the cells were arrested in the G1 phase of cell cycle. I found that cytokine deprivation for more than 15 hrs leads to an irreversible loss in their viability. When F7 and F7E2 cells were stimulated with IL-2, IL-3 or EPO, the cells synchronously began to replicate their DNA after 6 hrs, showed a maximum percentage of cells in S phase after 12 hrs, began to divide 15 hrs later, and the most of cells had reentered G1 18 hrs later. In the case of A15 cells, although the cells also synchronously progressed the cell cycle, it has taken longer time for one round of cell cycle. However, no such change was observed in IL-3-stimulated A15 cells. This observation may reflect the deficiency of apparent tyrosine phosphorylation in A15 cells due to the lack of association between IL-2Rβ and a *src*-family kinase(s). On the other hand, when BER2 cells were stimulated by EGF, the cells begin to enter S phase within 6-9 hrs, but fail to progress to G2/M phase.

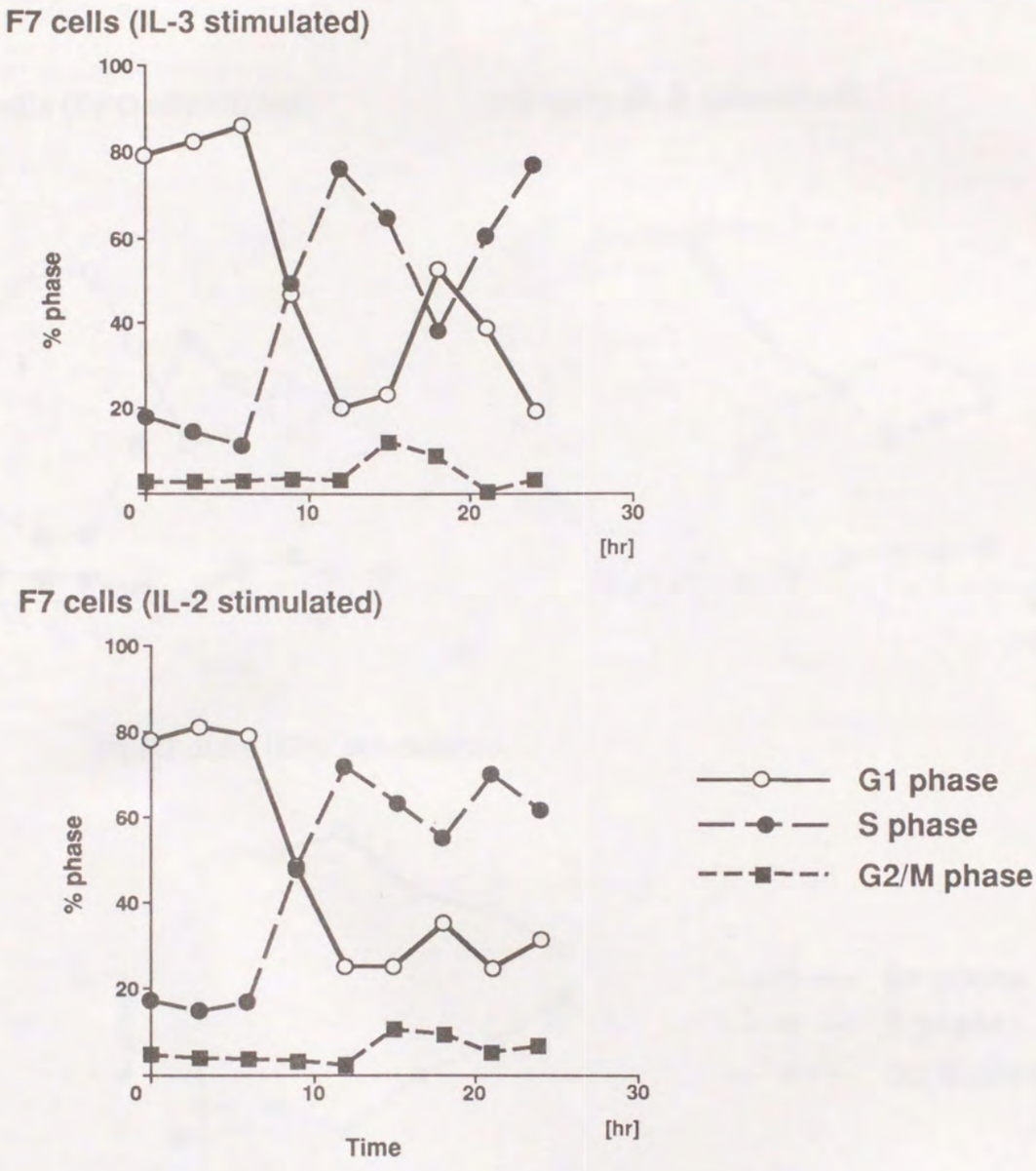
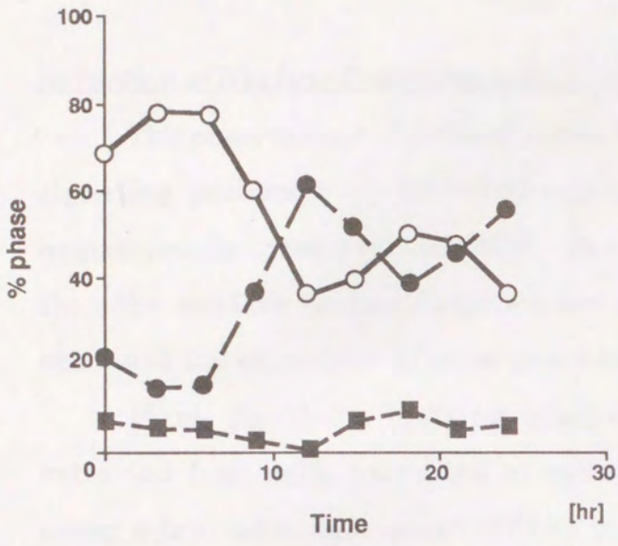


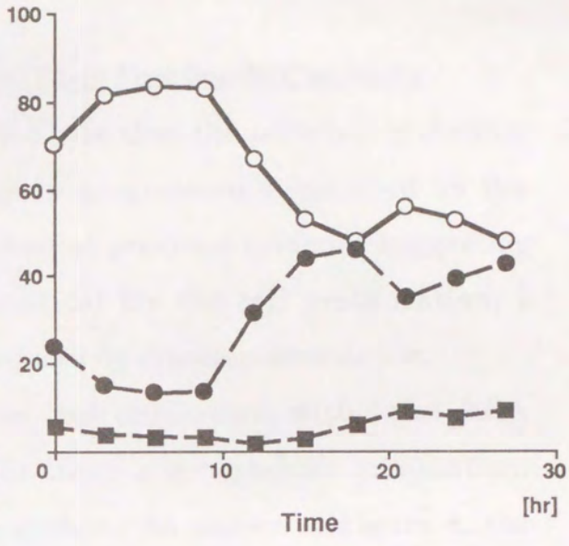
Figure 3. Cell Cycle Analysis of BAF-B03-derived Cells.

F7, F7E2, A15 and BER2 cells were synchronized by growth factor starvation and stimulated with cytokines (F7cells by IL-3 and IL-2; F7E2 cells by EPO; A15 cells by IL-2; BER2 cells by EGF). Cells were harvested at various times after starvation, stained with propidium iodide, and analyzed by flow cytometry as described in EXPERIMENTAL PROCEDURES. The calculated percentages of each phase are plotted.

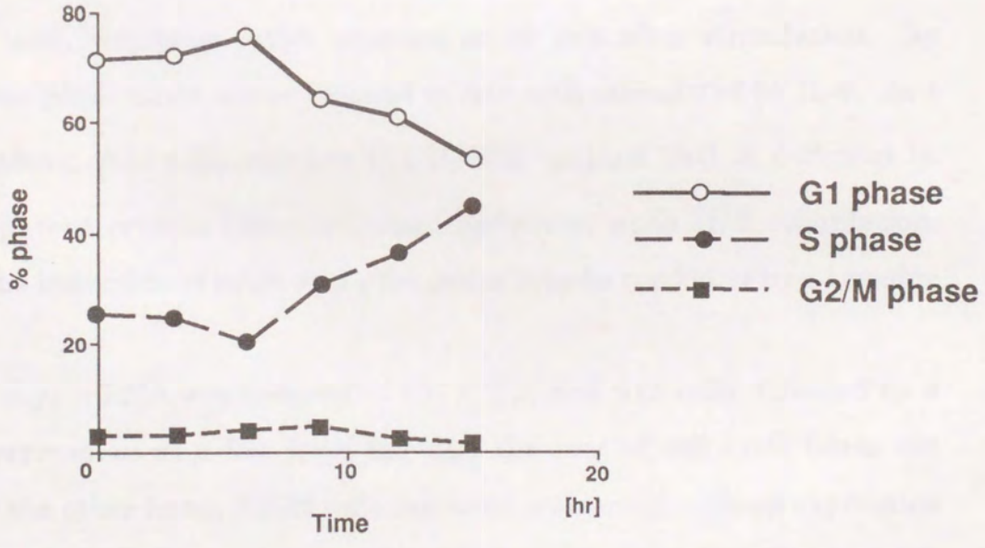
F7E2 cells (EPO stimulated)



A15 cells (IL-2 stimulated)



BER2 cells (EGF stimulated)



As I mentioned above, these cells could not proliferate thereafter, and died without cell division. This property was also observed in another BAF-B03 derived clone, BER10, similarly expressing EGFR (data not shown). Thus, EGF can induce G1/S transition, but not G2/M in BAF-B03 cells.

Induction of Nuclear Proto-Oncogenes and Their Families by Cytokines

The observations described above indicate that the presence of distinct signaling pathways for BAF-B03 cell cycle progression stimulated by the hematopoietic cytokines and EGF. In view of previous evidence suggesting that the nuclear proto-oncogenes are critical for the cell proliferation, I examined the expression of these genes induced by cytokine stimulation.

First, Northern blotting analysis was performed with total RNA extracted from cells harvested at various times after cytokine stimulation, using c-*jun*, c-*fos*, and c-*myc* cDNAs as probes. As shown in Figure 4, the accumulation of mRNA of c-*jun* and c-*fos* genes were rapidly induced in IL-2-stimulated F7, IL-3-stimulated F7, EPO-stimulated F7E2, and EGF-stimulated BER2 cells, with maximum levels attained at 10 min after stimulation. By contrast, these genes could not be induced in A15 cells stimulated by IL-2. As I mentioned above, A15 cells express the IL-2Rβ mutant that is deficient in inducing apparent protein tyrosine kinase activation upon IL-2 stimulation. Therefore, the induction of c-*jun* and c-*fos* genes may be mediated by a tyrosine kinase(s).

The c-*myc* mRNA was induced in F7, F7E2, and A15 cells, followed by a persistent expression at a low level through the rest of cell cycle (data not shown). On the other hand, BER2 cells manifest a severely reduced expression of c-*myc* even after EGF stimulation. The deficiency in c-*myc* induction, which was observed only in BER2 cells, appears to reflect the inability of the cells to enter mitosis, suggesting the c-*myc* induction may have an essential role for G2/M transition of BAF-B03 cells.

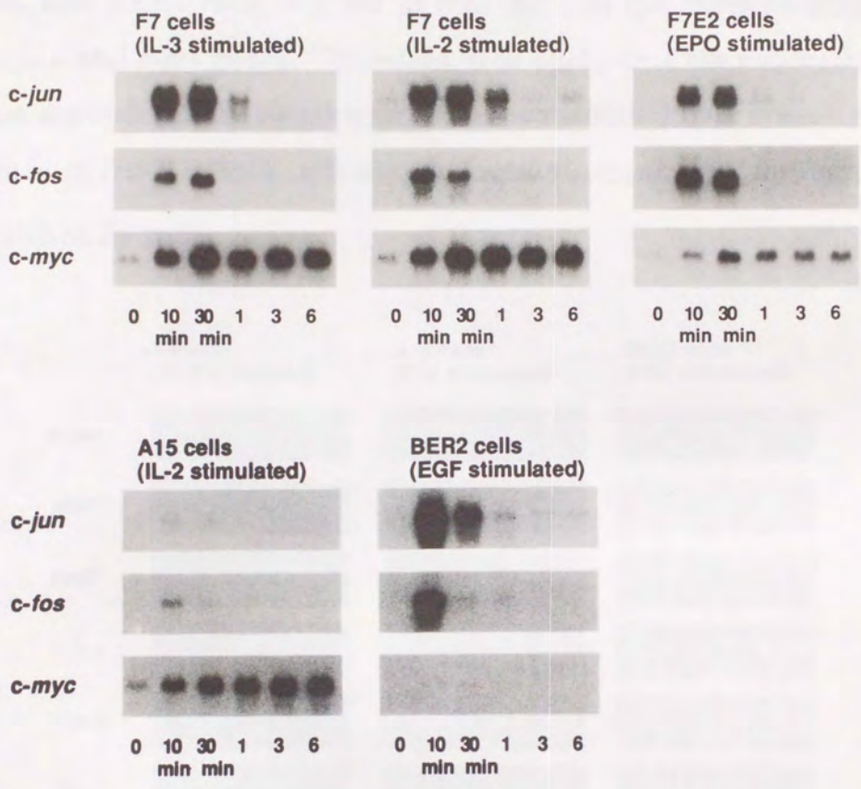


Figure 4. Differential Expression of c-*jun*, c-*fos* and c-*myc* mRNAs Induced by Cytokines in BAF-B03 Cells.

Northern blot analysis of total RNA extracted from cytokine-stimulated cells (F7 cells by IL-3 or IL-2; F7E2 cells by EPO; A15 cells by IL-2; BER2 cells by EGF) was performed as described in EXPERIMENTAL PROCEDURES. Membranes were exposed by autoradiography for 8 hrs (c-*myc*) or 24 hrs (others).

Next, RNA blotting analysis was performed to examine the expression of other nuclear proto-oncogenes and their families. The results are summarized in Figure 5. The expression of *junD*, c-*myb* and MAX are constitutive in the all of the cells. The *junB* and *fosB* genes were inducible in F7, F7E2, and BER2 cells, but not in A15 cells, in the same properties as the case of c-*jun* and c-*fos* genes. Therefore, it is likely that the expression of these two genes are induced by a pathway(s) that is mediated by a tyrosine kinase(s). In the case of *fra*-1 mRNA, efficient induction occurs only through the wild-type IL-2Rβ in F7 cells.

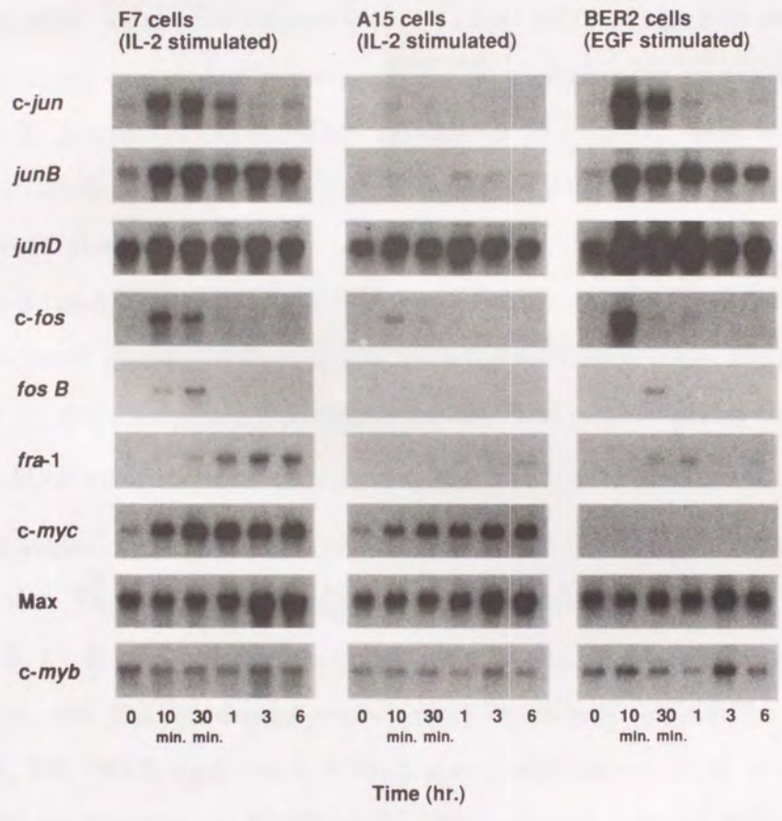


Figure 5. Expression of Nuclear Proto-Oncogenes and Their Families in Cytokine-Stimulated BAF-B03 Cells.

Norther blot analysis of total RNA extracted from cytokine stimulated cells (F7 cells by IL-2; A15 cells by IL-2; BER2 cells by EGF) was performed essentially by the same procedures as for Figure 4. Membranes were exposed for 4 days (*fra-1*), 8 hrs (c-*myc*) and 24 hrs (others).

Induction of Cell Cycle-Related Genes by Cytokines

Evidence has been provided that cyclins and cdks have critical roles for cell cycle progression. The induction of these genes might be influenced by the expression of nuclear proto-oncogenes either directly or indirectly. Therefore, I tried to examine which of these genes can be induced in the BAF-B03-derived cell lines by cytokine stimulation. Blotting analysis was performed, and the results are summarized in Figure 6.

Most of the cyclin mRNAs were similarly induced by IL-2, IL-3, and EPO except for cyclin D1, and the same was true for cdks mRNAs. Cyclin D1, which has been reported as a macrophage-specific cyclin (Matsushime et al., 1991), and cdk5 were not expressed in these cells (data not shown). It is interesting that cyclin D3 mRNA,which is specifically expressed in IL-2-dependent T lymphoid cells (Matsushime et al., 1991), was constitutively expressed in this cell line, and further induced not only by IL-2 but also by others. These observations suggest that cytokine-induced signaling mediated by IL-2, IL-3, and EPO receptors are very similar in terms of the induction of cell cycle-related genes. The mRNAs of all genes that were induced in IL-2-stimulated F7 cells were also expressed in the A15 cells bearing the mutant IL-2Rβ Upon IL-2-stimulation. However, the mRNA induction kinetics of the genes were significantly delayed, correlating with the longer time required for A15 cell cycle. Significantly, in BER2 cells, the mRNA induction levels for the cyclins A, B, C, E and cdc2 were lower than those in F7, F7E2 and A15 cells. Accordingly, the EGF-induced signal may be linked to mRNA induction of cyclins D2, D3, cdk2 and cdk4, which are involved in G1/S transition, and induce DNA replication in BER2 cells. By contrast, the mRNA induction for the cyclin A, B and cdc2 involved in the G2/M transition of cell cycle was significantly low or undetectable in BER2 cells. This observation was correlated with the defect of G2/M transition in EGF-stimulated-BER2 cells.

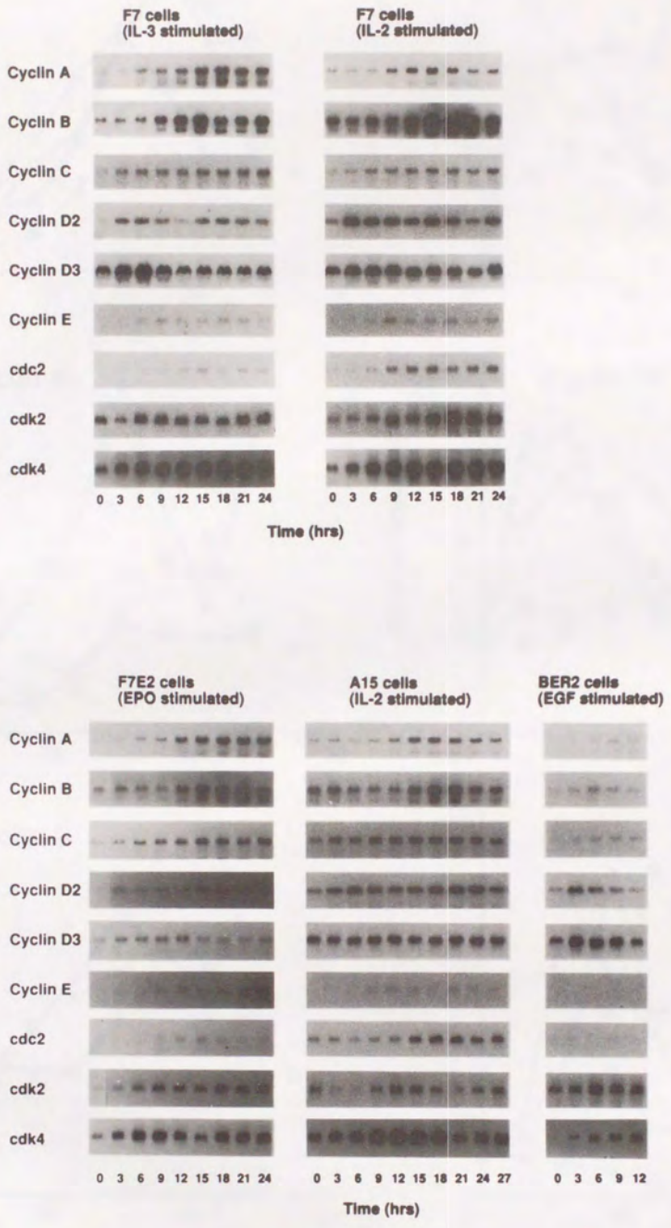


Figure 6. Differential Expression of Cyclins and cdks in BAF-B03-Derived Cells Stimulated by Cytokines.

(A) Northern blot analysis of cyclins and cdks expression was performed by the same procedures as for Figure 4. Filters were exposed by autoradiography for 7 days (cyclin E), 4 days (cdc2) or 24 hr (others).

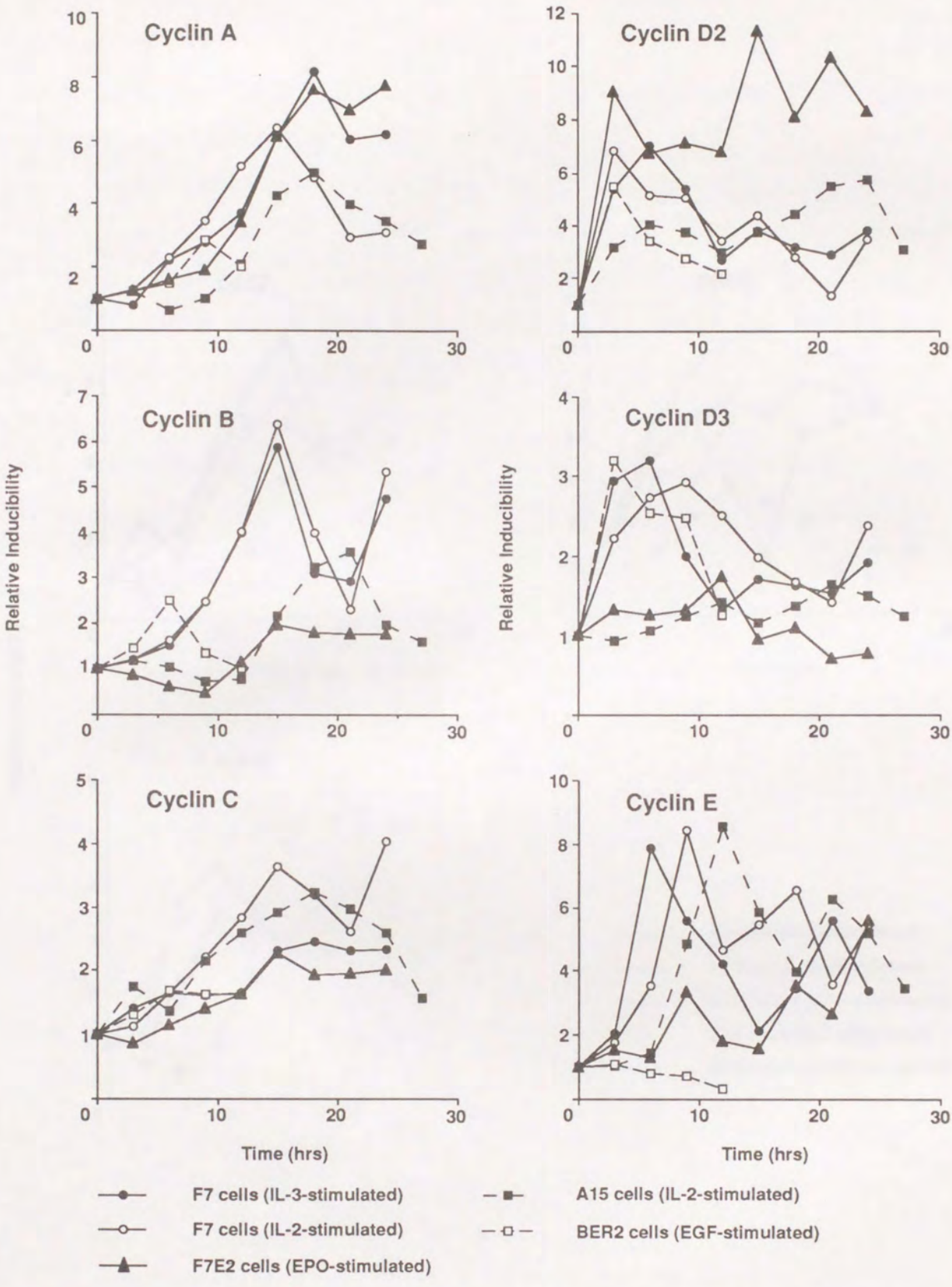
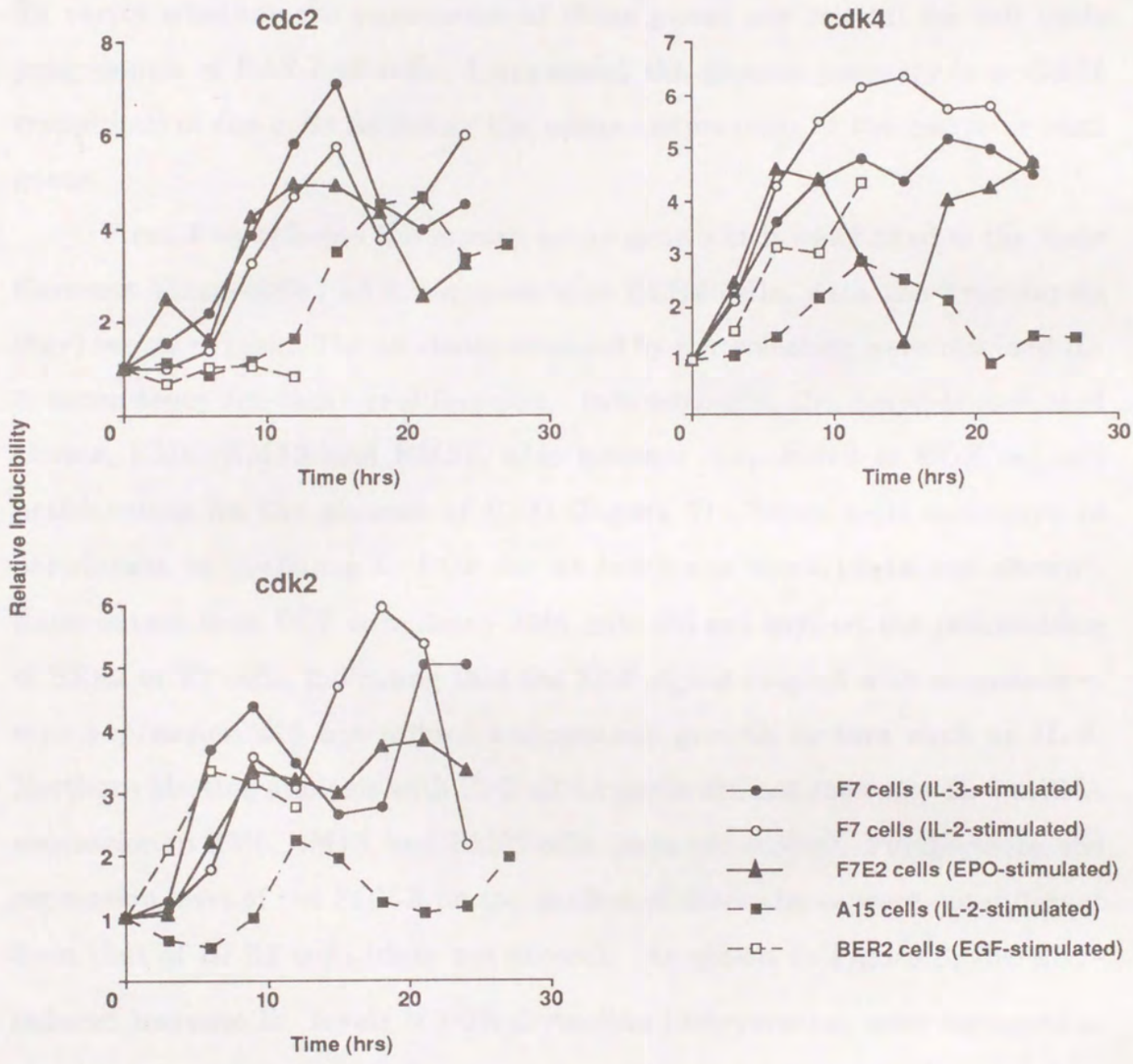


Figure 6-(B). Autradiogram in (A) was quantitated by densitometer scanning, and relative inducibilities were plotted in graphs.


cdc2
cdk4
cdk2
Relative Inducibility
Time (hrs)
Time (hrs)
Time (hrs)
F7 cells (IL-3-stimulated)
F7 cells (IL-2-stimulated)
F7E2 cells (EPO-stimulated)
A15 cells (IL-2-stimulated)
BER2 cells (EGF-stimulated)

Rescue of An Impaired Cell Cycle Progression in EGF-Stimulated BER2 Cells by Ectopically Expressed c-myc Gene

As described above, the ability of the BAF-B03 cells to undergo G2/M transition correlates with the induction of c-*myc*, cyclin A, B and cdc2 genes. To verify whether the expression of these genes are critical for cell cycle progression of BAF-B03 cells, I examined the growth property (e.g. G2/M transition) of the cells following the ectopic expression of the c-*myc* or cdc2 genes.

First, I transfected the human c-*myc* gene which was linked to the Rous Sarcoma Virus (RSV) LTR sequence into BER2 cells, with the hygromycin (*hgr*) resistant gene. The all clones obtained by *hgr* selection were retained IL-3 dependency for their proliferation. Interestingly, the c-*myc*-transfected clones, BM6, BM13 and BM21, also became responsive to EGF for cell proliferation (in the absence of IL-3) (Figure 7). These cells continued to proliferate in response to EGF for at least one week (data not shown). Supernatant from EGF-stimulated BM6 cells did not support the proliferation of BER2 or F7 cells, indicating that the EGF signal coupled with exogenous c-*myc* expression did not induce endogenous growth factors such as IL-3. Northern blotting analysis with IL-3 cDNA probe did not show any IL-3 mRNA expression in BM6, BM13, and BM21 cells (data not shown). Furthermore, the expression level of the EGF-R on the surface of these clones were not different from that of BER2 cells (data not shown). As shown in Figure 8, the EGF-induced increase in levels of [^{3}H]-thymidine incorporation were comparable between BER2 and BM6 cells , suggesting that the c-*myc* gene expression in BM6 cells did not alter the levels of DNA synthesis.

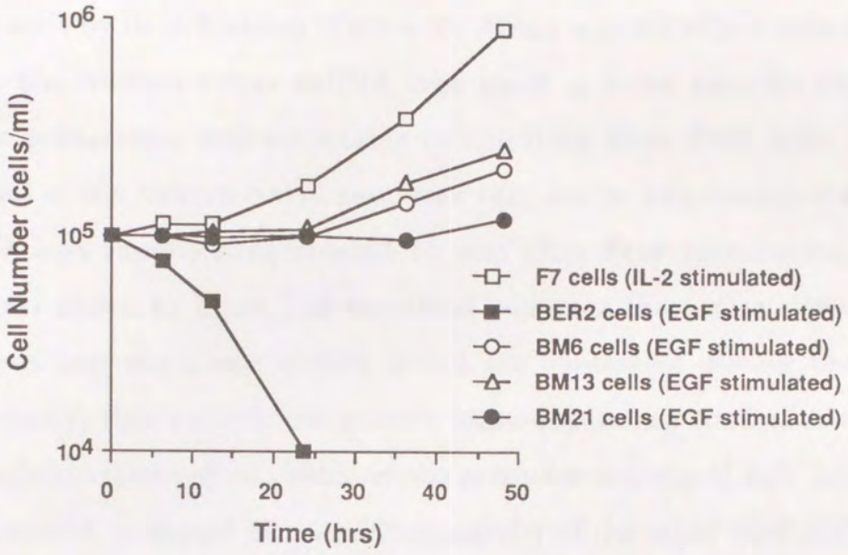


Figure 7. Proliferation Profiles for The Factor-Stimulated F7, BER2, BM6, BM13 and BM21 Cells.

Synchronized cells were plated at 1.0×10^5 cells/ml in the presence of IL-2 (F7 cells) or EGF (others). At the times shown, the density of viable cells was counted and represented on a logarithmic scale.

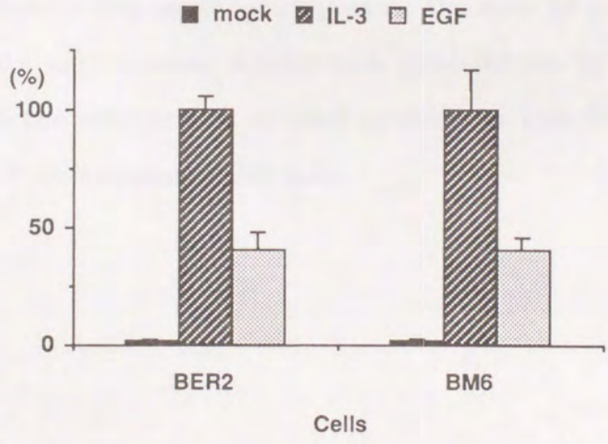


Figure 8. [^{3}H]-Thymidine Incorporation of BER2 and BM6 Cells.

Synchronized cells were assessed for their ability to incorporate [^{3}H]-thymidine in response to IL-3 or EGF. The actual incorporation levels after IL-3 stimulation are 17,276 ± 899 c.p.m. for BER2 cells and 20,029 ± 3,604 c.p.m. for BM6 cells. The data are the average of triplicate determinations.

Next, I examined the expression of the human c-*myc* gene and other genes in BM6 cells by RNA blotting (Figure 9). When a probe which selectively hybridizes to the human c-*myc* mRNA was used, a band specific for the human c-*myc* transcripts was detectable in the RNA from BM6 cells. The expression level of the human c-*myc* gene was very low in unstimulated state, but the mRNA was rapidly accumulated 10 min after EGF stimulation, and declined several fold 1 hr later, but remained constant thereafter. It is not clear at present how the c-*myc* mRNA levels are controlled during the cell cycle. Presumably, this reflects the growth state-dependent control of the c-*myc* mRNA stability (Dean et al., 1986) or the promoter activity of RSV LTR, or both. In this regard, it should be noted that activity of the other viral LTRs or the enhancer elements are also affected during the cell cycle (Imbra and Karin, 1986; Nabel and Baltimore, 1987; Doi et al., 1989b). Similarly, c-*myc* gene induction was also assessed in clones BM13 and BM21 (data not shown). Moreover, the mRNAs for cyclin A, B and cdc2 genes, whose expression were not observed in BER2 cells, were induced in BM6 cells upon EGF-stimulation (Figure 9).

On the other hand, I also established the clones which express the human cdc2 cDNA by the same strategies as the case of c-*myc*. As a result, I could not obtain any clones which can proliferate in response to EGF, suggesting that the expression of cdc2 protein is insufficient for the G2/M transition of EGF-stimulated BER2 cells.

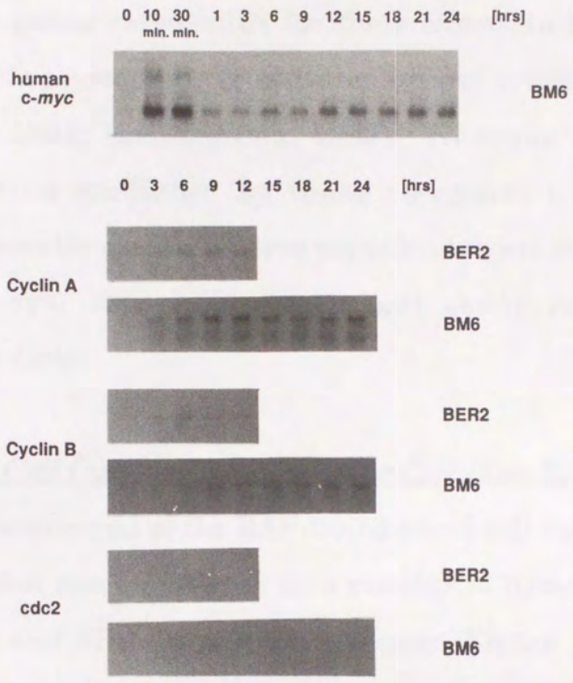


Figure 9. Expression of The Transfected Human c-*myc* Gene and Endogenous cdc2, Cyclin A and B Genes.

Total RNA extracted from EGF-stimulated BER2 and BM6 cells was subjected to Northern blot analysis essentially by the same procedures as for Figure 4. The RNA from EGF-stimulated BER2 cells was not analyzed after 12 hrs, due to the significant loss of viable cells beyond that point.

DISCUSSION

The hematopoietic cytokines induce the growth- and differentiation-signals through specific binding to the respective receptors, and induce the expression of the genes responsible for their effects in hematopoietic cells. Most of the cytokine receptors constitutes a new cytokine receptor family (Miyajima et al., 1992; Hatakeyama, 1993). However, the mechanisms of signal transduction mediated by these receptors have not been well understood. The results presented here provide insights into cytokine-induced signaling pathways, especially the target genes responsible for the hematopoietic cell cycle.

Cytokine-Induced Cell Cycle in Hematopoietic Cell Line, BAF-B03

The cell cycle analysis of the BAF-B03-derived cell lines revealed that the cell cycle progression can be induced by a number of hematopoietic cytokines, such as IL-2, IL-3 and EPO, in a similar manner (Figure 3). It has been also reported that the BAF-B03 cells can proliferate in response to other hematopoietic cytokines, including IL-6 and G-CSF, in cells expressing the respective receptors by cDNA transfection (Hibi et al., 1990; Fukunaga et al., 1991). Since the analysis of the target genes induced by these cytokines in this study did not allow us to distinguish the effects of the growth signaling, it is likely that an identical or closely-related signaling pathway(s) exist at least in part among these cytokine receptors in BAF-B03 cells. Although the cytoplasmic domains of these members of cytokine receptor superfamily are considerably diverged and do not have significantly conserved motifs, several amino acid stretches with some similarities are observed among the cytoplasmic regions of IL-2Rβ, IL-3R, EPOR, IL-6R(gp130) and G-CSFR (Hatakeyama, 1993; Minami et al., 1993a). Furthermore, the segment of IL-

2Rβ which is indispensable for the IL-2-induced proliferative signal transduction exhibits high degree of homology with the corresponding region of EPOR (Hatakeyama et al., 1989; D'Andrea et al., 1989b) (Figure 1). Accordingly, the closely-related signal(s) may be transduced via these conserved regions of the cytokine receptors. The biological effects of each cytokine are known to function in the specific types of cells. If the proliferative signals by these cytokines are transmitted via common pathway(s), the cell-specificity of each cytokine may be mainly regulated by the specific expression of ligands or receptors rather than by the signaling mechanisms stimulated by ligand/receptor interactions.

The results obtained from cell cycle analysis of A15 cells suggest that the coupling of the IL-2Rβ with a *src*-family kinase(s) is not essential for the proliferation of BAF-B03 cells. A15 cells which express the IL-2Rβ mutant lacking the "acidic" region of its cytoplasmic region were fail to induce apparent protein tyrosine phosphorylation upon IL-2 stimulation (Minami et al., 1993b). This defect may reflect in a slower progression of cell cycle in A15 cells compared to F7 cells (Figure 3).

On the other hand, EGF can induce the DNA synthesis in BER2 cells, but not mitosis. Essentially identical results were obtained in normal bone marrow cells and another IL-3-dependent cell line, IC2, expressing the exogenous EGFR by cDNA transfection (Rüden and Wagner, 1988; Wang et al., 1989). These observations suggest that the critical signals for G2/M transition of hematopoietic cells could not be transduced by EGFR, although the signals involved in triggering the proliferation of fibroblastic cells are also to some extent effective in hematopoietic cells.

The Existence of at least Two Distinct Signaling Pathways Mediated by IL-2Rβ

The analysis of nuclear proto-oncogene induction provide evidence for the presence of at least two distinct pathways for the IL-2Rβ-mediated signaling (Figure 10). One appears to be mediated by a tyrosine kinase(s) and linked to the induction of c-*jun*, *junB*, c-*fos* and *fosB* genes. Another pathway is linked to the induction of c-*myc* gene.

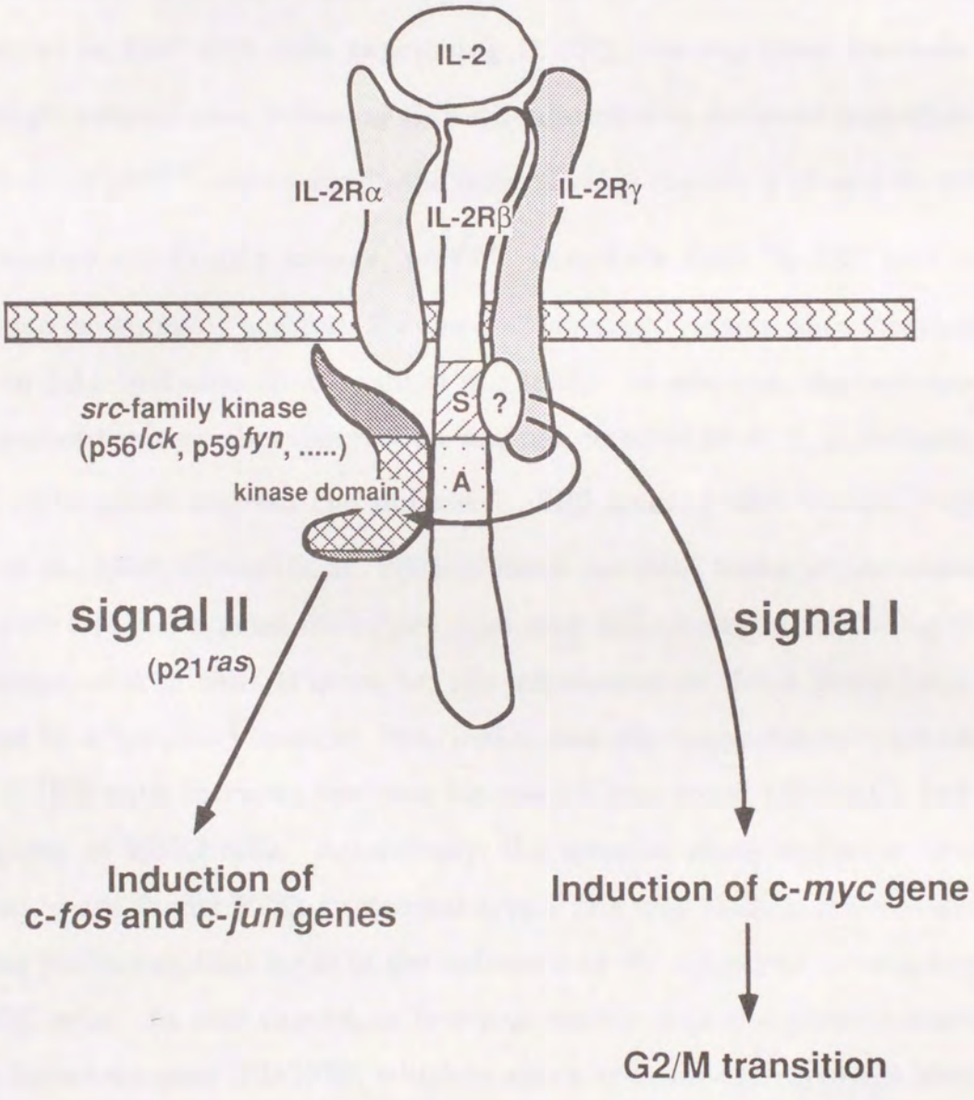


Figure 10. IL-2R-Mediated Signaling Involves at least Two Distinct Pathways.

1) Tyrosine Kinase-Mediated Pathway

As I mentioned previously, it has been shown that the "acidic" region of the cytoplasmic region of IL-2Rβ can interact with a sequence within the the kinase domain of $p56^{lck}$ PTK that is conserved among the members of the *src*-family PTK. Recently, it was also shown that the association of $p56^{lck}$ with IL-2Rβ is required for activating $p56^{lck}$ PTK and for inducing cellular tyrosine phosphorylation upon IL-2 stimulation (Minami et al., 1993b). As described previously, in BAF-B03 cells expressing IL-2Rβ, the apparent increase in tyrosine phosphorylation following IL-2 stimulation was observed regardless of the absence of $p56^{lck}$ expression in the cells. In this regard, it should be noted that another *src*-family kinase, $p59^{fyn}$, interacts with IL-2Rβ and this interaction presumably accounts for the IL-2-induced tyrosine phosphorylation events in BAF-B03 cells (Kobayashi et al., 1993). In addition, the increase in the apparent tyrosine phosphorylation was not detected upon IL-2 stimulation of A15 cells which express the mutant IL-2Rβ lacking this "acidic" region (Satoh et al., 1992; Minami et al., 1993b). Since our RNA blot analysis revealed that c-*jun* and c-*fos* gene induction was also not observed following IL-2 stimulation of A15 cells (Figure 4), the expression of these genes may be mediated by a tyrosine kinase(s). This notion was also supported by the finding where EGFR with intrinsic tyrosine kinase activity could efficiently induce these genes in BER2 cells. Accordingly, the tyrosine phosphorylation events mediated by *src*-family PTKs or receptor type PTKs may share a closely-related signaling pathways, that leads to the induction of the c-*jun* and c-*fos* genes in BAF-B03 cells. In this regard, it is worth noting that the platelet-derived growth factor receptor (PDGFR), which is also a receptor-type tyrosine kinase, can interact with a *src*-family PTK (Kypta et al., 1990). If the EGFR can also

couple with a *src*-family PTKs, the induction of c-*jun* and c-*fos* genes by IL-2Rβ or EGFR may be transduced via *src*-family PTKs.

Recently, it has been shown that IL-2-stimulation induces conversion of $p21^{ras}$ from GDP-bound form (inactive form) to GTP-bound form (active form) in BAF-B03-derived cells (Satoh et al., 1992). The activation of $p21^{ras}$ was not observed in the IL-2-stimulated A15 cells, suggesting that the $p21^{ras}$ may be a downstream signaling molecule of a *src*-family tyrosine kinase(s). On the other hand, the analysis of transcriptional activation of c-*fos* gene induced by IL-2 stimulation indicated that SRE element of c-*fos* promoter region is assumed to be the primary target for the IL-2-mediated signal(s) (Trouche et al., 1991; Hatakeyama et al., 1992).

Since the nuclear proto-oncogenes such as c-*jun* and c-*fos* were originally identified as the cellular homologue of retroviral oncogenes responsible for malignancies of cells (Curran et al., 1982; Maki et al., 1987), it was thought that the expression of these genes might be involved in the regulated-induction of the genes which are critical for cell cycle progression. However, the expression of the cell cycle-related genes in A15 cells was unaffected despite of the lack of c-*jun* and c-*fos* gene induction. This observation suggests that the induction of c-*jun* and c-*fos* genes is not required for the proper expression of cell cycle-related genes in BAF-B03 cells. Similar observations were also reported in the case of the signal transduction mediated by CSF-1R (Roussel et al., 1991). Although CSF-1 could not induce the proliferation of the NIH 3T3 cells expressing the mutant CSF-1R when Tyr residue at 809 was replaced with Phe, CSF-1R(Phe809), the efficient expression of c-*fos* and *junB* genes was observed in this cells. However, it is possible that the expression at a very low level of these proto-oncogenes may be sufficient for the induction of cell cycle-related genes. Furthermore, since the delay of cell cycle-related gene expression in BAF-B03 cells may reflect the deficiency of

these gene induction upon IL-2 stimulation, the expression of c-*jun* and c-*fos* genes may bear in part the regulation of cell cycle progression. At this time, a possible role of the c-*jun* and c-*fos* gene induction in the cell cycle regulation is still unclear .

2) Another Pathway Linked to c-myc Induction

As depicted in Figure 3 and 4, the ability of the BAF-B03-derived cells to progress their cell cycle, especially G2/M phase, correlates with the induction of c-*myc* gene. Since the defect in proliferative response of EGF-stimulated-BER2 cells can be rescued by an ectopic expression of c-*myc* gene, the pathway that linked to c-*myc* gene induction appears to be critical for cellular proliferation, in particular, G2/M transition of the cell cycle. In addition, the similar results, indicating a critical role of c-*myc* gene induction in cell proliferation, was also reported in the case of the growth signaling mediated by CSF-1R (Roussel et al., 1991). The NIH 3T3 cells expressing the mutant CSF-1R(Phe 809) were unable to grow in medium containing CSF-1. Although the activation of its intrinsic kinase activity and the induction of c-*fos* and *junB* genes were observed following CSF-1-stimulation of this cell, the induction of c-*myc* gene was impaired in this cells. However, the enforced expression of an exogenous c-*myc* gene restored their ability to proliferate in response to CSF-1. Hence, in addition to the IL-2-induced proliferation of the hematopoietic BAF-B03 cells, the c-*myc* expression is also critical for the CSF-1-induced proliferation of NIH 3T3 cells. It is not clear at present whether c-*myc* plays a role in G2/M transition of the NIH 3T3 cells.

BAF-B03 cells expressing the mutant IL-2Rβ lacking the "serine rich" region (Figure 2), also failed to progress their cell cycle upon IL-2 stimulation. In this cell line, any gene induction of the nuclear proto-oncogenes including c-*jun*, c-*fos* and c-*myc* were not observed (data not shown), indicating the critical signaling pathways, that leads to c-*myc* gene induction, may be transduced via the "serine rich" region of IL-2Rβ.

Inability of the c-*myc* gene induction correlates with the deficiency in the induction of certain cell cycle-related genes involved in G2/M transition such as cyclins A and B, and cdc2, suggesting that c-*myc* may function as an upstream cellular molecule required for these gene induction, thereby regulating them either directly or indirectly. In fact, their inducibility was restored by the ectopic expression of c-*myc* gene. Accordingly, the c-*myc* induction can trigger the expression of cell cycle related-genes such as cyclins A and B, and cdc2, and induce the G2/M transition of BAF-B03 cell cycle.

Mechanisms Involved in c-myc Gene Induction

As I mentioned above, it appears that a signal(s) derived from the "serine rich" region of IL-2Rβ couples to the c-*myc* gene induction, since the deletion or point mutation of this region totally abolishes c-*myc* gene induction (Hatakeyama et al., 1989b; Mori et al., 1991). In the case of CSF-1R, the Tyr-809 in their cytoplasmic region is involved in this signaling pathway, because the c-*myc* gene induction is abrogated in cells expressing CSF-1(Phe809) mutant (Roussel et al., 1991). This mutant receptor is fully functional in terms of CSF-1-induced tyrosine autophosphorylation, implying that the phosphorylated Tyr-809 may be coupled with an important signal transducer(s) that mediates the induction of c-*myc* gene. Thus, it is reasonable to think that the Tyr 809 of CSF-1 receptor and the "serine rich" region of IL-2Rβ may be functioning in an analogous manner.

Although considerable efforts have been made for a decade to characterize the transcriptional regulation for c-*myc* gene expression, the mechanisms of the regulation is still unclear. However, Langer et al. has reported recently that the enforced expression of the nuclear factor, *ets*-2, can also rescue the proliferative ability of NIH 3T3 cells expressing the CSF-1R(Phe809) mutant (Langer et al., 1992). Furthermore, introduction of the *ets* mutant lacking the transactivation domain suppressed the endogenous c-*myc*

expression in cells bearing the wild-type CSF-1R (Langer et al., 1992). These results suggest that the *ets* family member regulates the induction of c-*myc* gene either directly or indirectly.

The Role of c-myc in Cell Cycle Progression

Recent studies indicate that c-*myc* modulates the DNA replication (Lüscher and Eisenman, 1990; Hunter, 1991). Indeed, treatment with the c-*myc* antisense oligonucleotides can suppress the entry into S phase of the mitogen-stimulated peripheral blood lymphocytes (PBL) (Furukawa et al., 1990). On the other hand, my results suggested a novel role of c-*myc* in G2/M transition. However, our results by no means exclude the possibility that c-*myc* also plays a role in the G1 to S transition. It is possible that a very low level of c-*myc* induction after EGF stimulation of BER2 cells is sufficient for the cells to enter S phase.

What is the role for c-*myc* in G2/M transition? The primary structure of c-*myc* suggests that c-*myc* may function as a transcriptional regulator (Dang, 1991; DePinho et al., 1991). In fact, it has become evident that c-*myc* can bind to the specific DNA sequences by forming complex with MAX (Blackwood et al., 1992). Therefore, it is possible that the c-*myc* may regulate the expression of the other genes which are critical for G2/M transition of cell cycle. In this study, although the progression into G2/M phase of BAF-B03 cells were observed at 15 hrs (F7 and F7E2 cells) or 18 hrs (A15 cells) after cytokine stimulation, the accumulation of c-*myc* mRNA was observed immediately after stimulation, reached a peak at 1 hr (F7 and F7E2 cells) or 3 hrs (A15 cells). It is also worth noting that certain levels of c-*myc* mRNA persist for the rest of the cell cycle (data not shown). The similar kinetics for human c-*myc* gene induction were also observed in the EGF-stimulated BM cells that are capable of proliferating in response to EGF by exogenous c-*myc* gene expression (Figure 9). Although it is not clear yet whether the rapid

accumulation of c-*myc* mRNA after stimulation or persistent expression of c-*myc* mRNA through the cell cycle, the identification of the target genes for c-*myc* will allow us to address this issue further. The data obtained in this study suggests the possibility that cyclins A, B and cdc2 genes may be the target genes of c-*myc*. Recently, the genomic gene of cdc2 was isolated and the promoter region was identified (Dalton, 1992). However, the direct involvement of c-*myc* in expression of cdc2 gene has not been examined yet.

It has been shown that the deregulation of c-*myc* gene by translocation or amplification was linked to the malignancies of various cells (Dang, 1991; DePinho et al., 1991). More recently, it has been reported that the enforced expression of c-*myc* in the G1-arrested cells results in the programmed cell death, apoptosis (Askew et al., 1991; Evan et al., 1992; Shi et al., 1992). In my study, the expression level of human c-*myc* gene among the three different BM clones were almost comparable (data not shown), indicating that the fine-tuning of c-*myc* gene expression may be required for the proper cell cycle progression induced by cytokines.

On the other hand, it has been reported that the N-terminal domain of c-*myc* can physically associate with pRB *in vitro* (Rustgi et al., 1991). This finding suggests a possible role of c-*myc* other than its transcriptional activity.

Redundant Expression of Cell Cycle-Related Genes

The cell cycle progression is known to be regulated by the kinase(s) that are composed of cdks and cyclins. However, the mechanisms underlying cell cycle regulation by them have not been well understood. The data presented in this study suggests that cyclins A and B, and cdc2 and may have an important role for G2/M transition of BAF-B03 cell cycle, because the expression of these genes correlates well with the progression of G2/M phase. On the other hand, the expression of cell cycle genes involved in G1/S transition were redundant in BAF-B03 cells. Interestingly, it has been recently reported that cyclin D1

and D3 can form complexes with cdk4, and cyclin D-cdk4 complex can phosphorylate the retinoblastoma gene product (pRB) and an RB-like protein (p107) (Matsushime et al, 1992), which are the potential regulators of cell cycle . It is possible that the cyclin D3-cdk4 complex can function in BAF-B03 cells because mRNA of these genes were expressed in all transfectants including EGF-stimulated BER2 cells. The mRNA of cdk2 were also induced in BER2 cells, however, the catalytic partners of cdk2, cyclin A and E, were not induced in this cells, suggesting the expression of cdk2 may simply reflect the redundancy. Alternatively, an unidentified cyclin(s) may cooperate with cdk2 to progress G1/S. Further investigation will be required to elucidate the mechanism of cell cycle progression by cyclins and cdks.

In conclusion, my experimental approach may have opened a way to clarify the nature of signaling pathways by cytokines which involve the induction of nuclear proto-oncogenes and their families as well as cyclin and cdk genes, and may have pointed to the essential role for c-*myc* in the cell cycle progression.

EXPERIMENTAL PROCEDURES

Cell and Cell Culture

The BAF-B03, a subclone of BA/F3, is a bonemarrow-derived murine IL-3 dependent pro-B cell line (Palacios and Steinmetz, 1985). It exhibits the following profile of cell surface marker expression: Mac-1^+, $Bp1^-$, $B220^-$, $sIgM^-$, $Lyb1^-$, $Thy1^-$, $Ly1^-$, $L3T4(CD4)^-$, $Lyt2(CD8)^-$ and IL-$2R\alpha^+$ (Hatakeyama et al., 1989). F7 and A15 are BAF-B03-derived stable transfectant clones expressing wild-type human IL-2Rβ and IL-2Rβ mutant lacking the internal "acidic region", respectively (Hatakeyama et al., 1989). F7E2 is also a BAF-B03-derived clone which was established by transfection of human EPOR cDNA into F7cells (Hatakeyama et al., 1992). BER2 is also a BAF-B03-derived clone which was obtained by transfecting the plasmid in which human EGF-R cDNA was inserted into pNeoSRαII (kindly provided by Dr. Atsushi Miyajima, DNAX Research Inst. Palo Alto, CA; Wang et al.,1989). Clones BM6, 13 and 21 were obtained by transfecting the human c-*myc* expression plasmid, pN-LTRmyc, (containing the second and third exons of genomic human c-*myc* gene; Battey et al., 1983) into BER2 cells. Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS) and 10% (v/v) conditioned medium from the WEHI-3B cell line (10% WEHI conditioned medium) as a source of IL-3.

Cells were synchronized in G1 phase by depriving of cytokines for 15 hrs. To analyse cell cycle progression and gene expression, cells were stimulated with IL-3 (10% WEHI conditioned medium) for F7 cells, recombinant human IL-2 (2 nM) for F7 and A15 cells, recombinant human EPO (2 nM) for F7E2 cells, and recombinant human EGF (10 ng/ml) for BER2 and BM cells.

DNA Transfection

Plasmid DNAs were transfected into the cells by an electroporation procedure as described previously (Doi et al., 1989a). Selection was initiated 24 hrs after the transfection, using 2 mg/ml G418 for BER2 cells, or 1 mg/ml hygromycin for BM cells in 10% WEHI conditioned medium. Cells were cultured in 24-well microculture plates. Drug resistant colonies were picked up, and subsequently cloned by limitting dilution as described previously (Hatakeyama et al., 1989).

Cell Cycle Analysis

Essentially, the analysis was done by following the protocol provided by CycleTEST™ , Becton Dickinson (Vindeløv et al., 1983). Briefly, cells were suspended in citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 5% DMSO) (2.5×10^6 cells/ml) and treated with trypsin to solubilize cell membranes. After mixing for 10 min at room temperature, the solution containing trypsin inhibitor and Ribonuclease A was added. The solutions were again mixed at room temperature, and after 10 min ice-cold solution containing propidium iodide was added to stain the nuclei (final propidium iodide concentration; 125 μg/ml). Fluorescence intensity in each cell nucleus was measured with a FACScan™ (Becton Dickinson). Approximately 20,000 cells were analyzed from each sample at a rate of 50 to 100 cells per second. The percentages of cells in the each phase of the cell cycle were determined by analysis with the computer program CellFIT™ (Becton Dickinson).

Preparation of Probe DNA

The probe DNAs for cdc2, cdk2, c-*jun*, *junB*, *junD*, c-*fos*, *fra*-1, c-*myc* and c-*myb* mRNAs were prepared as follows: cdc2, 1.6 kbp EcoRI fragment excised from pcdc2 which contains human cdc2 cDNA (Ninomiya-Tsuji et al.,

1991); cdk2, 1.5 kbp EcoRI fragment from the plasmid in which human cdk2 cDNA is inserted into pBluescript II SK(+) (Ninomiya-Tsuji et al., 1991); c-*jun*: 1.0 kbp FspI/HincII fragment from pSP-c-jun (Rauscher et al., 1988); *junB*: 0.5 kbp EcoT14I/SacI fragment from the plasmid which contains mouse *junB* cDNA in pBluescript KS(+) (Hirai et al., 1989); *junD*: 0.9 kbp SphI fragment from the plasmid which contains mouse *junD* cDNA in pBluescript(+) (Hirai et al., 1988); c-*fos*: 0.4 kbp StuI/SacI fragment from pSV-mFOS (Hatakeyama et al., 1992); *fra*-1: 260 bp KpnI/BstXI fragment from pSP-fra1 (Cohen and Curran, 1988); c-*myc*, 1.9 kbp HindIII fragment from pMc-myc54 (Stanton et al., 1983); c-*myb*, 2.0 kbp EcoRI fragment from pcmybE which EcoRI segment of human c-*myb* gene is inserted into pBR322 vector (Franchini et al., 1983). The plasmids containing c-*jun*, *junB*, *junD* and *fra*-1 cDNAs were kindly provided from Dr. Iba (Inst. of Medical Science, University of Tokyo, Japan). The cdk4, Cyclins A, B, C, D2, D3, E, Max and *fosB* cDNAs were obtained by PCR cloning method using the synthesized primers from the published cDNA sequences (cdk4; Matsushime et al., 1992: cyclin A; Wang et al., 1990: cyclin B; Pines and Hunter, 1989: cyclin C and E; Lew et al., 1991: cyclin D2 and D3; Matsushime et al., 1991: Max; Prendergast et al., 1991: *fos*B; Zerial et al., 1989), and the cDNAs were each subcloned into the HincII site of pUC19 vector. The cDNA fragments excised from these plasmids were used as probes. To detect the expression of human c-*myc*, 1.5 kbp ClaI/EcoRI fragment from third exon of pN-LTRmyc was used as a human c-*myc* specific probe (Dmitrovsky et al., 1986).

RNA Extraction and Northern Blot Analysis

Total cellular RNA from each cells was prepared by denaturation in guanidium thiocyanate followed by pelleting through a CsCl cushion (Shibuya et al., 1990). For northern blot analysis, 10 μg of total RNA was electrophoresed on 1% agarose formaldehyde gels, and transferred onto nylon membranes.

Probes were labeled with [α^{32}P]-dCTP using a Multiprime Labeling Kit (Amersham) and hybridized as described previously (Harada et al., 1990). Specific activity was approximately 1×10^6 c.p.m./ng for all the probe DNAs.

Measurement of The Cell Number Increase

Synchronized cells were cultured at a density of 1×10^5/ml (5 ml of culture) in RPMI-1640 supplemented with 10% FCS containing recombinant human IL-2 (2 nM) (F7 cells) or recombinant human EGF (10 ng/ml) (BER2 and BM cells). Viable cell counts were determined by trypan blue staining.

Measurement of [^{3}H]-Thymidine Incorporation

Cells (2×10^4) were cultured without growth factors in RPMI-1640/10% FCS in a 96-well microculture plate for 15 hrs. After stimulation with recombinant human IL-2 (2 nM) or recombinant human EGF (10 ng/ml), cells were pulsed with 1 μCi of [^{3}H]-thymidine for 4 hrs prior to harvest. [^{3}H]-thymidine incorporation was measured as described previously (Hatakeyama et al., 1989).

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