Interleukin-7 induces N-myc and c-myc expression in normal precursor B lymphocytes

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Expression of both N-myc and c-myc is induced rapidly and dramatically in normal pre-B cells after stimulation with interleukin-7 (IL-7), a pre-B cell-specific growth factor. These IL-7-induced increases in N-myc and c-myc expression are mediated at the transcriptional level; for N-myc, a major portion of the induction results from release of an attenuation block between exons 1 and 2. Although c-myc expression has been shown to be regulated by many factors, these studies provide the first demonstration of N-myc regulation by a growth factor. Furthermore, mitogen-induced proliferation of more mature B-lineage cells is accompanied by induction of c-myc expression in the complete absence of N-myc expression. Thus, N-myc expression in normal B-lineage cells, as in transformed cells, is limited to cells that represent the precursor stages of this differentiation pathway. Together, these findings imply a specific function for N-myc in the early stages of B-cell development.

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The myc family of cellular proto-oncogenes has three well-characterized members, the c-myc, N-myc, and L-myc genes; these genes have a similar three-exon structure and encode related but distinct nuclear phosphoproteins (for review, see Zimmerman and Alt 1990). The function of myc proteins is believed to involve regulation of transcription, DNA replication, or, possibly, both (for review, see Lüscher and Eisenman 1990). Because myc genes have distinct expression patterns in normal development and changes in expression correlate with response of cultured cells to differentiation agents and mitogenic stimuli, they may function to regulate gene expression in the context of cell growth and differentiation (for review, see Zimmerman and Alt 1990). This speculation is supported by the fact that c-myc has been found to bind a specific DNA sequence (Blackwell et al. 1990), and regions conserved among myc proteins include DNA–protein or protein–protein interaction motifs found in a number of DNA-binding, transcriptional regulatory, and cell-lineage determination proteins (for review, see Collum and Alt 1990; Lüscher and Eisenman 1990).

Expression of c-myc occurs in most normal and transformed cell types and generally parallels proliferative state of the cell, nondividing cells usually express low levels of c-myc, which are rapidly induced by proliferative stimuli. Regulation of c-myc expression has been shown to occur at several levels, including transcriptional initiation, transcriptional attenuation, and post-transcriptional processes (for review, see Zimmerman and Alt 1990). Expression of N-myc and L-myc is limited to various undifferentiated cell types; expression becomes differentially restricted in a lineage- and stage-specific fashion as development proceeds (Zimmerman et al. 1986; Maugrauer et al. 1988; Downs et al. 1989). In situ studies suggest that for several tissues, N-myc expression may correlate better with the cellular differentiated rather than the proliferative state (Maugrauer et al. 1988). The unique structural and expression characteristics of myc proteins suggest distinct physiological functions.

Developmentally regulated expression of N-myc has been implicated in the context of the B-lymphocyte lineage (for review, see Zimmerman and Alt 1990). Primary B-cell differentiation occurs in the bone marrow of adult mammals (for review, see Alt et al. 1987). During this process, precursor B [pre-B] cells first assemble and express immunoglobulin heavy-chain genes and subse-
frequently assemble and express immunoglobulin light-chain genes. Complete immunoglobulin molecules are deposited on the cell surface leading to the generation of the next major B-cell differentiation stage, the surface immunoglobulin-positive B lymphocyte. The surface immunoglobulin-positive cells then migrate from the bone marrow to peripheral organs such as the spleen, where stimulation [with antigen or mitogen] can lead to further division and maturation into immunoglobulin-secreting cells. Pre-B cell lines express the N-myc gene, whereas lines that represent more differentiated B-lineage cells do not [Zimmerman et al. 1986]. In contrast, c-myc expression occurs in lymphoid cell lines representing all B-cell differentiation stages [Zimmerman et al. 1986] and is induced by mitogen stimulation of normal splenic B cells [Kelly et al. 1983].

We have employed a normal pre-B-cell culture system to assay mechanisms that regulate N-myc and c-myc expression in normal precursor B lymphocytes. Such a system involves culturing bone marrow cells in a manner that provides for the growth and maintenance of pre-B cells. In these lymphoid bone marrow cultures, growth of precursor B lymphocytes is dependent on the lymphokine interleukin-7 [IL-7] that is produced by stromal cells [Namen et al. 1988a,b]. IL-7 induces proliferation and is required for survival of normal bone marrow pre-B cells but does not support further differentiation [Lee et al. 1989; Sherwood and Weisman 1990]. We now demonstrate that IL-7 rapidly induces N-myc and c-myc expression in normal pre-B cells and characterize the molecular mechanisms by which this induction occurs.

Results

**Induction of gene expression in bone marrow pre-B cells by IL-7**

To examine the effect of IL-7 on specific gene expression in pre-B cells, we assayed RNA from freshly isolated and IL-7-treated pre-B cells for hybridization to a number of specific DNA probes (see below). For this purpose, we purified surface immunoglobulin-negative, B220 antigen-positive cells from total bone marrow; the B220 antigen is expressed specifically by cells of all stages of the B-cell lineage [Kincade et al. 1981]. Thus, this selected cell population is comprised largely of pre-B lymphocytes. An aliquot of sorted pre-B cells was harvested for RNA preparation immediately after sorting. The remaining cells were cultured for 2 days in the presence or absence of 500 U/ml of IL-7, the concentration shown to cause maximal proliferation of purified pre-B cells [Lee et al. 1989].

Purified pre-B cells maintained in media alone for 2 days had decreased viability, and no RNA was recovered; therefore, Northern analyses were performed only with the untreated and IL-7-treated cells [Fig. 1]. Comparison of the relative hybridization intensities of N-myc and c-myc probes to that of a glyceraldehyde phosphate dehydrogenase [GAPDH] control probe indicated that N-myc expression increased approximately eightfold, and c-myc expression increased about twofold in the pre-B cells treated with IL-7 for 2 days as compared with untreated cells [Fig. 1, lane 1 vs. 2]. Thus, IL-7 treatment of normal bone marrow pre-B cells generates a population of cells with increased N-myc and c-myc expression. These experiments imply strongly that the observed increase results from a direct effect of IL-7 on myc gene expression but do not eliminate unequivocally the possibility of an acute selection for a subset of cells that express N-myc and c-myc at high levels.

Large-scale IL-7-dependent lymphoid cell cultures

A more detailed analysis of the mechanism responsible for the apparent IL-7 induction of myc gene expression required a large number of IL-7-responsive cells. The quantity of pre-B cells necessary is not obtained readily by standard cell-sorting techniques; furthermore, transformed pre-B cell lines constitutively express high levels of N-myc and c-myc [Zimmerman et al. 1986] and do not respond measurably to exogenous IL-7 [data not shown]. Therefore, we used long-term lymphoid bone marrow cultures (LBMCs), as an enriched source of pre-B cells [Whitlock and Witte 1982; Whitlock et al. 1985]. LBMCs are derived by culturing total bone marrow; in these cultures, stromal cells adhere and produce a feeder layer capable of supporting growth of nonadherent cells that represent various B-cell developmental stages. To obtain a sufficient number of IL-7-responsive cells, we first established LBMC for at least 4 weeks and then cultured these cells further in the presence of IL-7 [500 U/ml]. LBMCs were maintained in the presence of IL-7 until cells reached a density of ~10^6 nonadherent cells per milliliter (~1 week of culture). The nonadherent cells were then removed from the stroma and analyzed further as described below.
To characterize the cells in the IL-7 cultures, we assayed the population for rearrangements of immunoglobulin \( \mu \) heavy-chain \([J_\mu]\) and immunoglobulin \( \kappa \) light-chain \([L_\kappa]\) loci by assaying, respectively, EcoRI or EcoRI plus BamHI-digested genomic DNA for hybridization to \( J_\mu \) or \( J_\kappa \) probes (Coffman and Weissman 1983; Coleclough et al. 1983; Alt et al. 1984); the amounts of DNA assayed for each sample were controlled by hybridization to several probes that detect genomic restriction fragments that do not undergo rearrangement (e.g., \( C_\mu \), N-myc; see Materials and methods). The \( J_\mu \) probe detects a single 6.2-kb germ-line EcoRI fragment in unrearranged liver DNA (Fig. 2, lane 1), however, no detectable germ-line \( J_\mu \) fragment was present in genomic DNA from either IL-7 cultures or bacterial lipopolysacharride (LPS)-treated BALB/c spleen cells (polygonally activated B lymphocytes), although some prominent submolar rearranged fragments were obvious (Fig. 2, lanes 2,3). This finding indicates that, like normal splenic B cells, the cells in IL-7 cultures have rearranged both \( J_\mu \) alleles. In contrast, the 6.5-kb \( J_\kappa \)-hybridizing fragment in DNA from IL-7 cultures showed no detectable diminution in signal intensity compared with that of liver DNA (Fig. 2, lanes 4,5); unlabeled smaller bands are nonspecific cross-hybridizing fragments), whereas the intensity of the \( J_\kappa \) fragment in LPS-stimulated spleen DNA, as expected (Coleclough et al. 1983), was only 40% that of the control sample (Fig. 2, lane 6). Therefore, the majority of cells in the LBMC had rearranged their immunoglobulin heavy-chain genes but not their immunoglobulin light-chain genes. These findings are in accord with recent results of others who assayed cell clones or purified cells that had a similar dependency on IL-7 for proliferation (Hardy et al. 1991; Rolink et al. 1991). In addition, the IL-7 cultures also express significant levels of mature-size immunoglobulin \( \mu \) heavy-chain RNA, germ-line immunoglobulin \( \kappa \) RNA, and RNA from the pre-B cell-specific \( A_5 \) gene [not shown]. Together, these findings demonstrate that the bulk of the cells in the IL-7 cultures represent the pre-B stage of B-cell differentiation.

**Figure 2.** Rearrangement status of immunoglobulin loci in IL-7-cultured cells. Genomic DNA (10 \( \mu \)g) from mouse liver [lanes 1,4], lymphoid bone marrow cultures [lanes 2,5], and LPS-stimulated spleen cells [lanes 3,6] was digested with EcoRI [left] or EcoRI plus BamHI [right] and assayed by Southern blotting methods for hybridization to the \( J_\mu \) or \( J_\kappa \) probes, respectively. DNA markers [m] represent the \( \lambda \) phage HindIII fragments of 6.6, 4.4, 2.3, and 2.0 kb. Arrows indicate the positions of the germ-line (unrearranged) restriction fragment harboring each of the indicated genes.

**IL-7-regulated myc gene expression**

To examine IL-7 dependence of specific RNA transcript expression in IL-7 cultured cells, cells were washed with IL-7-free medium several times and grown in this media for 5 hr. Cells harvested after 5 hr in IL-7-free medium showed no change in cell viability or GAPDH RNA expression levels; therefore, observed changes in myc gene expression [see below] do not result from cell death or a general loss of steady-state RNA expression. Northern blotting analyses demonstrated that steady-state levels of N-myc and c-myc transcripts were reduced >10-fold after growth of cells in IL-7-free medium (Fig. 3, cf. lanes 1 and 3).

To examine the kinetics of IL-7 induction of N-myc and c-myc expression, cells grown in IL-7-free media [for 5 hr, as discussed above] were incubated in media either with or without IL-7 and harvested for RNA preparation after various times of incubation. Northern analyses revealed that treatment of cells with IL-7 for as little as 30 min resulted in a dramatic (>10-fold) induction of N-myc and c-myc RNA expression (Fig. 3, lane 7). Maximal expression of N-myc was induced after 30 min and was maintained over the course of 11 hr of treatment [Fig. 3 and data not shown]. Induction on c-myc transcripts occurred similarly, except that maximal induction occurred between 1 and 3 hr, after which levels gradually declined [Fig. 3 and data not shown]. This represents the first finding of growth factor induction of N-myc expression, whereas the induction profile for c-myc RNA expression is reminiscent of that observed in other systems (Kelly et al. 1983; Dean et al. 1987; Klemisz et al. 1989). Notably, expression of the c-myb (Fig. 3) and c-fos [not shown] genes was not detectably altered by the IL-7 treatment protocol, despite the fact that these genes have been shown to be responsive to other growth factors in other cell types [for review, see Rivera and Greenberg 1990, Shen-ong 1990]. Therefore, IL-7 treatment of pre-B cells appears to regulate expression of the N-myc and c-myc genes in a specific fashion.

To assay for physiological activity of the IL-7 treatment on the pre-B population, we measured \(^{3}H\)thymidine uptake to roughly estimate rates of DNA synthesis. Upon readdition of IL-7 to the cultures, increased \(^{3}H\)thymidine incorporation was evident within the first 6 hr of treatment and increased further over the next 12 hr (Fig. 4). Therefore, we find a correlation between the increased expression of the N-myc and c-myc genes and the onset of new DNA synthesis in the IL-7-treated cultures.

**IL-7 induction of N-myc expression is specific to the pre-B stage of B-cell development**

To determine whether N-myc induction is specific to the IL-7 response of pre-B cells, we examined the effects...
Figure 3. IL-7 induction of myc gene expression in LBMCs. Total RNA from LBMCs grown in the presence of IL-7 was assayed by Northern blotting methods for hybridization to the indicated probes. Odd-numbered lanes contain 10 μg of total RNA; even-numbered lanes contain a one-tenth dilution (1 μg) of the RNA in the preceding odd-numbered lane. (Lane 1) RNA from LBMCs propagated in the presence of 500 U/ml of IL-7, [lane 3] RNA from LBMCs after incubation in the absence of IL-7 for 5 hr, [lanes 5,9,13,17] RNAs from LBMCs propagated in the absence of IL-7 for an additional 0.5, 1.0, 3.0, and 6.0 hr, respectively, [lanes 7,11,15,19] RNAs from LBMCs propagated after readdition of IL-7 for 0.5, 1.0, 3.0, and 6.0 hr, respectively. Other details are as described in the legend to Fig. 1.

Transcriptional regulation of c-myc and N-myc genes by IL-7

To study the role of transcription in the regulation of N-myc and c-myc by IL-7, we performed nuclear run-on assays with treated and untreated populations. To obtain a sufficient number of cells (~10^6) for these analyses, nonadherent cells from LBMCs were grown (in roller bottles) in the presence of IL-7 for several weeks. For the steady-state analyses, IL-7–grown cells were washed and incubated in medium lacking IL-7 for 5 hr. Cells were then incubated in the presence or absence of IL-7 for the indicated amount of time, harvested, and nuclei were isolated. An aliquot of cells was taken before and after the 5-hr incubation to confirm that expected changes in steady-state N-myc and c-myc RNA levels had occurred [data not shown]. Nuclei from the various cell populations were incubated in the presence of [32P]UTP, and extended RNA was isolated and assayed for hybridization to filters containing either slot-blotted or Southern-blotted single- or double-stranded DNA fragments that represented different regions of the N-myc, c-myc, or control genes. The relative hybridization of the labeled RNA to particular fragments was visualized by autoradiography (Fig. 6A-C represents different experiments and experimental protocols as detailed below and in the legend) and quantitated by densitometry. Relative incorporation into RNA complementary to particular myc probes in different nuclei preparations was estimated by quantitating the signal for each probe relative to that of a control GAPDH plasmid (which was assumed to be unchanged by a given treatment). The experiments were performed four times with consistent results [Table 1].

We observed significant hybridization of RNA from nuclei of untreated cells to probes that represented the first exon (probes are diagramed in Fig. 6D) of both the N-myc and c-myc genes. In contrast, we detected little or no hybridization of RNA from untreated cells to fragments representing exon 2 or 3 of the N-myc or c-myc genes [Fig. 6A–C, Table 1, RPMI]. In most experiments, RNA from nuclei of IL-7–treated cells hybridized with a severalfold increased intensity to the first exon probes of the N-myc and c-myc genes (Fig. 6A–C, Table 1, IL-7), indicating that IL-7 treatment can induce a relatively modest increase in transcriptional initiation of these genes. More strikingly, RNA from the IL-7–treated nuclei hybridized with greatly increased intensity to probes that represented the downstream exons of the N-myc and c-myc genes [Fig. 6A–C]. For most experiments, the increase in hybridization to N-myc exons 2 and 3 after IL-7 treatment was much greater than 10-fold [Table 1], for c-myc, increased hybridization to the exon 2 probe (exon 3 was not tested) was also significant [Table 1].

No significant hybridization was observed to DNA that represented the sense strand of N-myc exons 1, 2, and 3 [Fig. 6C, Table 1], clearly demonstrating that the
transcription has, in some cases, been associated with differential utilization of the two promoters (Spencer et al. 1990). The murine N-myc gene has a single promoter from which transcription initiates heterogeneously over an ∼10-bp region (DePinho et al. 1986). To test for potential changes in promoter utilization, we used S1 nuclease protection methods to assay cellular RNA for its ability to protect a probe that extended 480 bp upstream and 180 bp downstream from the normal transcriptional initiation site. These analyses did not detect any differences in the region from which N-myc transcription initiated in IL-7-treated or control cells [data not shown].

Discussion

Regulation of N-myc expression in normal pre-B cells

We have employed IL-7-dependent lymphoid bone marrow cultures as a system to investigate the molecular mechanisms of IL-7-regulated myc gene expression in normal pre-B cells. We have confirmed the general results obtained with the IL-7 cultures by also using normal purified pre-B-cell populations, further validating the use of this culture system to explore detailed molecular regulatory mechanisms in normal pre-B cells.

We find that expression of both the N-myc and c-myc genes is modulated rapidly by the presence or absence of IL-7 in the growth medium of normal pre-B cells. Although induction of c-myc has been observed in many systems and in response to many factors (for review, see Zimmerman and Alt 1990), our studies represent the first example of growth factor-dependent regulation of N-myc expression. The IL-7 induction of N-myc and c-myc gene expression in pre-B cells is quite specific, as transcription and steady-state transcript levels from two

Figure 5. Induction of myc gene expression in LPS-treated splenocytes. Total splenocytes from 8-week-old BALB/c mice were disaggregated and incubated for 2.5 hr in medium that contained either 50 μg/ml of LPS, 500 U/ml of IL-7, or 50 μg/ml of LPS plus 500 U/ml of IL-7. Total RNA was prepared and assayed for hybridization to the indicated probes as outlined in Fig. 1. [Lane 1] RNA from total untreated spleen cells; [lane 2] RNA from LPS-treated spleen cells; [lane 3] RNA from IL-7-treated spleen cells; [lane 4] RNA from LPS + IL-7-treated spleen cells. RNA from the Abelson murine leukemia virus [A-MuLV]-transformed pre-B cell line 18-8 [lane 5, 1 μg; lane 6, 10 μg] was used as a positive control.
Figure 6. Transcriptional regulation of myc gene expression by IL-7. Nuclear run-on analyses were performed with nuclei isolated from cells grown for 5 hr in the absence of IL-7, followed by the addition of medium that either did (IL-7 panels) or did not (RPMI panels) contain IL-7. Labeled nascent transcripts were hybridized to double-stranded N-myc, c-myc, or GAPDH probes that were immobilized on nitrocellulose filters either by slot-blotting (A) or by Southern blotting after digestion with appropriate restriction endonucleases (B). For the double-stranded probe experiments, DNA fragments that contain the various N-myc or c-myc exons were liberated from the appropriate plasmids by digestion with the restriction enzymes indicated in D (exon probes). (C) Labeled nascent transcripts were also assayed for hybridization to immobilized single-stranded DNA probes that represent the indicated regions of the N-myc (A-D) and c-myc (probes E,F) genes. Both anti-sense (+) and sense (−) probes for each region were analyzed. The GAPDH probe used to control this experiment was double stranded.

other nuclear oncogenes, c-myb and c-fos, do not change under the conditions assayed. Within the B-cell lineage, induction of N-myc expression due to specific growth factor stimulation is limited to cells of the pre-B stage (or perhaps earlier) and does not occur in immunoglobulin-positive B cells. Thus, purified or cultured bone marrow pre-B cells respond to IL-7 stimulation with both increased N-myc and c-myc expression, whereas splenic B cells respond to mitogen stimulation with increased expression of only c-myc.

Expression of the c-myc gene has been shown to be regulated at multiple and varied levels, including transcriptional initiation, transcriptional attenuation, and post-transcriptional mechanisms such as differential RNA stability [for review, see Marcu 1987]. The N-myc gene has been shown to be regulated by transcriptional initiation [for review, see Zimmerman and Alt 1990; R. Smith, K. Zimmerman, G. Yancopoulos, A. Ma, and F.W. Alt., in prep.] and post-transcriptional mechanisms (Babiss and Friedman 1990). The N-myc gene also has been shown to be expressed specifically in transformed cells that represent the precursor but not the more mature stages of B-cell differentiation [Zimmerman et al. 1986]. In such cell lines, regulation occurs at the level of transcriptional initiation [Mc et al. 1991; R. Smith et al., in prep.]. In contrast, we find that the IL-7-induced increase in transcription of the N-myc and c-myc genes in normal pre-B cells results predominantly from alleviation of transcriptional attenuation. These findings represent the first report of transcriptional regulation through
full N-myc gene occurs in most cell types with steady-state RNA expression levels being regulated by post-transcriptional mechanisms (Babiss and Friedman 1990). Transcriptional attenuation of the c-myc, L-myc, c-myb and c-fos genes can occur between the beginning of exons 1 and 2, and in several cases, control of elongation through this region has been shown to contribute to regulated gene expression (Bentley and Groudine 1986; Bender et al. 1987; Nepvu et al. 1987; Krystal et al. 1988; Watson 1988; Lamb et al. 1990; Mechti et al. 1991; for review, see Rivera and Greenberg 1990; Spencer and Groudine 1990). A short portion of the first exons of the murine and human c-myc genes, which contains a potential stem-loop structure, has been shown to mediate a transcriptional block (Bentley and Groudine 1988; Miller et al. 1989; Wright and Bishop 1989). Consistent with our findings, transfection-based analyses of N-myc expression also have suggested the presence of a negative regulator of N-myc expression somewhere within the 3' end of exon 1 and the first intron (Hiller et al. 1991). The block to transcriptional elongation in the c-myc gene also may depend on which of the two c-myc promoters is used [Spencer et al. 1990]. The N-myc gene has a single major promoter [DePinho et al. 1986] that appears to be utilized to initiate transcription in both the presence and absence of IL-7.

In summary, our studies demonstrate that multiple mechanisms are differentially active to determine relative N-myc and c-myc expression levels during B-cell differentiation. In nonstimulated pre-B cells, transcription initiates at both the N-myc and c-myc promoters at a significant rate, but steady-state RNA levels are low as a result of termination of transcription before exon 2. Stimulation of pre-B cells with IL-7 leads to a rapid increase in steady-state N-myc and c-myc RNA levels largely as a result of relief of transcriptional attenuation. However, upon differentiation of pre-B cells to B cells, steady-state N-myc expression is completely down-regulated at the level of transcriptional initiation (R. Smith, K. Zimmerman, G. Yancopoulos, A. Ma, and F.W. Alt, in prep.) while the c-myc gene remains transcriptionally competent. The evolution of these distinct mechanisms to allow growth factor-regulated N-myc expression in pre-B cells and complete down-regulation of N-myc expression upon differentiation into B cells implies strongly that N-myc serves a necessary physiological function that needs to be acutely regulated in pre-B cells and is not required during the latter stages of B-cell development.

**The N-myc gene likely has a specific function in pre-B cells**

Deregulated expression of N-myc or c-myc has been suggested to lead to down-regulation [cross-regulation] of the expression of other myc family genes (for review, see Zimmerman and Alt 1990). However, studies of tumors that arise in mice that express a deregulated N-myc transgene suggested that such cross-regulation is indirect and may require additional events associated with the participation of a particular myc gene in the transformation process [Ma et al. 1991]. Clearly, we find a substantial increase in the expression of both the N-myc and c-myc genes upon the addition of IL-7 to pre-B cells; therefore, an inverse relationship in the expression levels of these genes is not necessarily present in normal cells. This finding implies that these two highly related genes might have unique functions within the same cell.

The expression patterns of the N-myc and c-myc genes observed in growth factor- or mitogen-stimulated normal pre-B or B cells are maintained in transformed cells that represent these stages [Zimmerman et al. 1986]. Therefore, the transformation mechanisms that act at
particular B-cell differentiation stages require and/or preserve the stage-specific expression patterns of these two related nuclear oncogenes. The precise physiological roles of myc family genes are unknown, although a large body of evidence indicates that they function as specific transcription factors in the context of cellular proliferation and/or differentiation [for review, see Lüscher and Eisenman 1990]. IL-7 stimulates pre-B-cell proliferation in the absence of differentiation [Lee et al. 1989]. Expression of c-myc has been implicated in the proliferation of most cells [for review, see Kelly and Siebenlist 1986]; and deregulated, high-level expression of the N-myc gene, in the absence of c-myc expression, can contribute to cellular transformation both in vitro and in vivo [for review, see Zimmerman and Alt 1990]. Therefore, the rapid stimulation of both N-myc and c-myc in response to IL-7 may imply a specific role for both genes with respect to pre-B-cell proliferation.

In normal cells or in cell lines in which N-myc is not implicated in the transformation process, N-myc expression has been correlated with differentiation stage rather than with cell proliferation [Finkelstein and Weinberg 1988; Mauagru et al. 1988; Thiele et al. 1988; Schmid et al. 1989]. Because N-myc is not expressed in most proliferating cells, c-myc appears sufficient to provide the “myc functions” in dividing cells. Thus, it is conceivable that N-myc expression also may be involved in processes distinct from proliferation. Such processes would include pre-B-cell survival, expression of pre-B-specific functions, or prevention of pre-B-cell differentiation.

Materials and methods

Cell purification

Femoral bone marrow cells of 8-week-old BALB/c mice [Jackson Laboratories, Bar Harbor, ME] were separated based on surface marker expression, as described by Lee et al. [1989]. The purified pre-B cells were either harvested immediately or grown in culture for 2 days in tissue culture media [RPMI-1640 supplemented with 5% fetal calf serum, 50 µg/ml 2-mercaptoethanol (2-ME), 50 µg/ml of penicillin and streptomycin] with or without the addition of recombinant IL-7 at 500 U/ml.

LBMCs

Long-term cultures were established basically as described by Whitlock and Witte [1982]. Femoral bone marrow cells from 5- to 8-week-old C57Bl/6 mice [Jackson Laboratories, Bar Harbor, ME] were suspended at 10^6 cells/ml of tissue culture media in 75 cm² Falcon T-flasks [Becton-Dickenson, Lincoln Park, NJ]. Cells were maintained by replacing one-half of the media weekly, taking care not to remove nonadherent cells. All experiments were performed with cultures that had been established for at least 4 weeks. The cells employed in the induction time course experiments were obtained by adding IL-7 to a concentration of 500 U/ml to the LBMCs. The cultures were grown for ~1 week, during which time the nonadherent lymphocyte population was expanded from 10^3 to 2 × 10^6 cells/ml. Cells were collected for analysis by gentle pipetting, removing as few stromal cells as possible. The cells were washed three times with media to remove IL-7 and grown in IL-7-free media for 3–5 hr before reinducing with IL-7 at 500 U/ml. No change in cell viability or number (as detected by trypan blue staining) was detected over the course of the experiment. The cells used in the nuclear run-on analysis procedure were prepared in a slightly different manner. The lymphocytes were purified from stromal cells after the initial growth in the presence of IL-7 by passing to a new flask in which the stromal cells were left to adhere overnight. The nonadherent cells were passaged for successive 2- to 3-day periods [leaving the stroma behind] until no stromal cells could be detected. The cells were then expanded in roller bottles with media containing IL-7 at 500 U/ml for 10–14 days until ~10^6 cells were obtained. At this time, cells were washed and incubated as described above and reinduced with IL-7 for 30 min or 5 hr before harvesting for nuclei preparations. During the period of stromal cell-independent propagation, cell number initially decreased but then rapidly increased as the IL-7 responding population proliferated. These IL-7-dependent cultures could be maintained for ~3 weeks, at which point cell viability dropped precipitously.

Nucleic acid analysis

DNA and RNA were prepared, electrophoresed, blotted, and assayed for hybridization to nick-translated fragments as described previously [Alt et al. 1984]. The following probes were used for Northern or Southern blotting analyses: for N-myc, the murine cDNA fragment M2.1 [Zimmerman et al. 1986], for c-myc, the murine cDNA fragment pMC2.1 [Zimmerman et al. 1986], for c-myb, the 2.5-kb R1 fragment [Bender and Kuehl 1986]; for c-fos, the 1.3-kb BglIl–PvuII fragment of FBJ-2 [Cullen et al. 1982]; for GAPDH, the rat cDNA fragment [provided by S. Chen Kian, Mt. Sinai Medical School, NY]; BI30GAPDH [Fort et al. 1985], for Cµ, the 900-bp PstI fragment from pABm-11 [Bothwell et al. 1981]; for Jµ, the 2.7-kb HindIII–Iµ-containing fragment [Blackwell et al. 1989], and for Jµ, the 1.9-kb BamHI–Iµ fragment [Blackwell and Alt 1984]. RNA samples were blotted onto Zeta probe [Bio-Rad, Richmond, CA], and the DNA samples were blotted onto nitrocellulose [Schleicher & Schuell, Keene, NH].

Nuclear run-on analysis

Isolation of nuclei, labeling of the nascent mRNA, and hybridization onto immobilized gene fragments were performed essentially as described by Lacy et al. [1989] or by Miller et al. [1989], with the following modifications: Cells were lysed with 0.25% NP-40, preincubation of the filters was done for 10–15 hr, after washing, the filters were treated with 10 µg/ml of RNAse in 2× SSC for 30 min at 37°C, the filters were then treated with 0.2 mg/ml of proteinase K for 1 hr at 37°C. Autoradiographs were analyzed with an LKB ultrascan XL. Each myc gene fragment signal was quantitated and normalized to the GAPDH signal. Other details are found in the legend to Figure 5.

Cell proliferation assay

Nonadherent lymphocytes were removed directly from LBMCs growing in tissue culture media. The cells were washed twice in media and plated in quadruplicate in 96-well microtiter plates at 5 × 10^4 cells/well in a total volume of 0.05 ml of RPMI. After the cells were incubated for 5 hr at 37°C, 0.05 ml of RPMI with 1000 U/ml of IL-7 was added to one-half of the cells and 0.05 ml of RPMI was added to the others. At this point, one set of quadruplicate wells for each the IL-7-incubated and IL-7-free cells was pulsed with 1 µCi of [3H]thymidine for 6 hr. After this incubation, the pulsed cells were harvested and a second set of
quadruplicate wells was pulsed with \[^{3}H\]thymidine for the subse-
quent 12 hr of incubation. Cells were harvested onto glass
fiber filters and washed extensively with 15% TCA, and incor-
porated radioactivity was quantitated by liquid scintillation
counting.

**Stimulation of splenic lymphocytes with bacterial LPS**

Spleen cells of 4-week-old BALB/c mice were introduced into
culture and treated as described by Yancopoulos et al. (1987)
with 50 μg/ml of bacterial LPS, with 500 U/ml IL-7, or with both for 2.5 hr.

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