MOLECULAR AND CELLULAR BIOLOGY, Feb. 1991, p. 854–863
0270-7306/91/020854-10$02.00/0
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Vol. 11, No. 2

Hyperexpression of Interleukin-7 Is Not Necessary or Sufficient for Transformation of a Pre-B Lymphoid Cell Line

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Received 13 September 1990/Accepted 5 November 1990

Interleukin-7 (IL-7) is a potent stimulator of pre-B-lymphocyte proliferation. Pre-B cells transformed by a variety of oncogenes including those of the ABL protein tyrosine kinase family were screened for endogenous IL-7 mRNA expression by polymerase chain reaction and a sensitive bioassay for secreted IL-7. Some v-abl but none of the BCR/ABL, v-*src*, v-*fms*, v-*myc*, v-*ras*, or v-*raf* transformants analyzed contained elevated IL-7 transcripts. None of the cell lines secreted detectable bioactivity. We overexpressed IL-7 via a retroviral vector in an IL-7-dependent pre-B cell line to assess the potential for autocrine growth stimulation and malignant transformation. We achieved dramatic deregulation of IL-7 translational suppression by removing portions of the 5′ flanking region. Levels of IL-7 expression much greater than those needed to establish factor-independent growth did not induce colony formation in agar by IL-7-expressing pre-B cell lines, and the majority of these lines were nontumorigenic in syngeneic mice. The same pre-B cell line transformed by v-abl displayed a highly malignant phenotype while containing dramatically lower IL-7 transcript levels. We conclude that endogenous IL-7 expression is not a necessary event in transformation of pre-B cells, nor is it sufficient to explain the malignant phenotype in v-*abl*-transformed cells. Up regulation of endogenous IL-7 expression in some transformed pre-B cells may be one of several synergistic events which can lead to malignant conversion.

Normal development of B-lymphoid cells requires factors secreted by bone marrow stromal cells. Interleukin-7 (IL-7), a potent cytokine for lymphoid cells at the pre-B stage of development, was recently purified and cloned from bone marrow stromal cells. It induces proliferation but not differentiation of pre-B cells (29, 38), and also has growth stimulatory effects on early and mature T-cells (6, 15, 37, 47). A receptor for IL-7 was recently cloned and shown to be expressed on pre-B cells, progenitors to pre-B cells, and some myeloid and T cells, but not on mature B cells (14, 40).

Acute lymphoblastic leukemia of both children and adults is predominantly a disease of lymphoid cells at the pre-B stage of development (17, 34). The proliferative effect of IL-7 on pre-B cells suggests that it could play a role in leukemogenesis if its normal signal transduction pathway was deregulated, amplified, or bypassed. This could occur by stromal-cell overproduction of IL-7, activating of mutations in the cytokine or its receptor, or expression in pre-B cells leading to an autocrine loop.

Murine pre-B lymphoid cells can be transformed by a wide variety of oncogenic agents and provide a model system for studying mechanisms of leukemogenesis. Retroviral oncoproteins which can transform pre-B cells include P140 v-fms, a transmembrane receptor (3); p21 v-ras, a cytoplasmic GTPase (18, 45); p74 v-raf, a serine-threonine kinase (42); and pp60 v-src, P85 v-fes, and P160 v-abl, cytoplasmic tyrosine kinases (18). In addition, the human chimeric BCR/ABL proteins P185 and P210, encoded from the Philadelphia chromosome, can transform murine pre-B cells (36). P185 BCR/ABL is commonly associated with acute lymphoblastic leukemia, and P210 BCR/ABL is associated with chronic myelogenous leukemia and acute lymphoblastic leukemia (26). The ability of multiple oncogenes to transform pre-B cells raises the possibility that a variety of initiating mechanisms can converge on a common growth signal.

In vitro models show that pre-B lymphoid cells transformed by oncogenes are initially dependent on stromal-cell support but later progress to fully transformed and factor-independent lines (48, 51). This suggests that transformation involves several steps, including activation or bypass of growth factor pathways. Activated ABL proteins have previously been shown to abrogate the requirements of a T-lymphoid line for IL-2 (8) and of myeloid lines for IL-3. In the case of IL-3, evidence for both bypass (7, 9, 41) and autocrine (16, 19) mechanisms has been described elsewhere.

In this report we have analyzed murine pre-B lymphoid cells transformed by *ABL* and other oncogenes for turn on of endogenous IL-7 transcription. We found that IL-7 is inappropriately expressed in some v-*abl* transformants but the level is not sufficient to explain the fully transformed phenotype. To directly test if autocrine stimulation by IL-7 is sufficient for transformation of pre-B cells, we hyperproduced IL-7 via a retroviral vector in a nontransformed pre-B lymphoid line. IL-7 dosage can be varied by removing portions of the 5′ nontranslated sequence. Pre-B cells expressing IL-7 can attain autocrine growth and secrete biologically active IL-7, but they rarely acquire a tumorigenic phenotype.

MATERIALS AND METHODS

Construction of IL-7 expression vectors and PCR analysis. Cytoplasmic RNA was prepared from bone marrow stromal and hematopoietic cells by urea lysis as previously described (45). All RNAs used for polymerase chain reaction (PCR) analysis were preanalyzed on ethidium bromide-stained formaldehyde-agarose gels to ensure intactness and to equalize amounts. The sequences of the oligonucleotide primers

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used to generate the murine IL-7 gene segments are as follows: primer 1, 5'TGTCCTGTTTATATACTGCCC3'; primer 2, 5'CAGACCATGTTCCATGTTTCT3'; primer 3, 5'CGGAAGTTATGGCAAAGCCAG3'; and primer 4, 5'AT ATCTGCTATGCTCTTTACT3'. For generating cDNA, 1 μg of RNA was incubated with 30 pmol of downstream primer 1 or 4; 1 mM (each) dATP, dCTP, dTTP, and dGTP; 5 mM dithiothreitol; 15 U of RNAsin (Promega); and 200 U of Moloney murine leukemia virus reverse transcriptase (BRL) in PCR buffer (50 mM KCl, 20 mM Tris hydrochloride [pH 8.4], 2.5 mM $MgCl_2$, 0.1 mg of bovine serum albumin per ml) in a total volume of 40 μl for 1 h at 37°C. The reverse transcriptase was inactivated by heating the reaction mixture to 95°C for 5 min. Upstream primer 2 or 3 (30 pmol) and 3 U of *Taq*I polymerase (Pharmacia) were then added in PCR buffer, giving a total volume of 100 μl. PCR was performed using 40 cycles of amplification in a Perkins-Elmer-Cetus machine with a 45°C annealing temperature and 30-s intervals and ramps. The reaction products were separated on a 1% agarose gel, and the DNA bands were either electroeluted for subcloning or subjected to Southern blotting for endogenous IL-7 expression analysis.

IL-7 DNA fragments were subcloned by ligating appropriate linkers to blunted ends and inserting them into the *Pst*I site of pcDL-SRα296, a COS cell expression plasmid (46), or into the *Hin*dIII site of pMV6TKneo, a retroviral expression plasmid (24). Southern blotting was performed with alkaline transfer to Zeta probe membranes (Bio-Rad). Blotting, probe hybridization, and washing conditions were as suggested by the manufacturer. Blots were probed with the internal IL-7 oligonucleotide 27-mer 5'ATGACAGGAACTGATAGTAA TTGCCCG3', which was end labeled by nucleotide transfer from [γ-^{32}P]ATP with T4 polynucleotide kinase (Pharmacia). The specific activity was approximately 5×10^8 cpm/μg of DNA.

Transfection of fibroblasts and COS cells. Fibroblasts were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal calf serum and 5% calf serum. To generate helper-free IL-7 retrovirus, psi-2 murine packaging fibroblasts were transfected either by $CaPO_4$ precipitation with 5 μg of plasmid DNA or by electroporation with 20 μg of plasmid DNA. G418-resistant colonies were expanded and screened for virus production as described previously (35).

COS monkey kidney cells were transfected by a DEAE-dextran-chloroquine method. Briefly, subconfluent monolayers in 10-cm dishes were incubated for 4 h at 37°C in 4 ml of DMEM containing 50 mM Tris hydrochloride (pH 7.4), 400 μg of DEAE-dextran (Sigma Chemical Co.) per ml, and 5 μg of pcDL-SRα(IL-7) plasmid. Cells were rinsed with phosphate-buffered saline and incubated for 3 h in DMEM containing 2% fetal calf serum, 0.58 mg of L-glutamine per ml, penicillin-streptomycin (Gibco), and 100 μM chloroquine (Sigma). Monolayers were rinsed in phosphate-buffered saline and refed with recovery medium (DMEM, L-glutamine, penicillin-streptomycin, and 5% fetal calf serum). COS cells were biochemically analyzed or IL-7-containing supernatant was harvested on day 3 posttransfection.

Infection of lymphoid cultures. v-abl- and P210 BCR/ABL-transformed lymphoid bone marrow cultures were derived by infecting BALB/c femoral bone marrow with Abelson murine leukemia virus or pJW-RX virus (35), respectively, and plating the cells under long-term lymphoid culture conditions as described previously (45). v-src-, v-myc-, v-raf-, v-myc–v-raf-, and v-Ha-ras-transformed lymphoid cultures were a gift from Jacalyn Pierce (National Cancer Institute). The v-fms-transformed line was a gift from Gary Borzillo (2) (St. Jude Children's Hospital).

The IL-7-dependent pre-B lymphoid line was derived by single-cell cloning of nonadherent cells from a long-term lymphoid bone marrow culture (20). IL-7-dependent pre-B cells were maintained either on bone marrow stromal feeder layers or in 0.2% culture fluid from COS cells containing an IL-7 expression plasmid. These cells were infected with Abelson murine leukemia virus, P210 BCR/ABL, or P185 BCR/ABL retrovirus (36); MMCVneo v-myc retrovirus (45); v-Ha-ras and c-fos retroviruses (gifts from Naomi Rosenberg [Tufts University]); or IL-7 retroviruses by suspending cells at a concentration of 5×10^5/ml of virus-containing culture fluid with 8 μg of Polybrene per ml and incubating them for 3 h at 37°C. Viral culture fluids contained approximately 5×10^5 to 5×10^6 G418-resistant CFU/ml. Following infection, cells were suspended in fresh medium at a density of 5×10^5/ml. All lymphoid cells were grown in lymphoid medium (RPMI 1640 medium supplemented with 5% fetal calf serum and 5×10^{-5} M-2-mercaptoethanol).

Metabolic labeling and immunoprecipitation. [^{35}S]methionine labeling and preparation of detergent lysates of cellular and secreted proteins were as described previously (51). Lysates were immunoprecipitated with a polyclonal rabbit antiserum directed against an extreme C-terminal 12-amino-acid peptide of murine IL-7, which was kindly provided by Frank Lee (DNAX Co.). The procedures for immunoprecipitation, polyacrylamide gel electrophoresis, and autoradiography were as previously described (25).

Bioassays. To measure IL-7 bioactivity, culture fluid was harvested from cells, filtered to remove cells, frozen and thawed once, and then added to 10^5 IL-7-dependent pre-B cells in 1 ml of lymphoid medium at the concentrations indicated in the figures. Viable cell numbers were determined on day 5 by trypan blue exclusion.

Formation of colonies in semisolid medium was measured by plating 10^5 cells per 6-cm plate in 10 ml of growth medium containing 15% fetal calf serum and Noble agar (Difco) as described previously (31).

To test for IL-7-independent growth, pre-B cells were switched to medium lacking COS cell-generated IL-7 at a density of 10^5 cells per ml 1 week postinfection. For viruses inducing factor-independent growth, approximately 2 weeks of maintainance were required before a uniformly viable culture was regenerated. Alternatively, since all vectors used here contain a *cis*-linked neomycin resistance gene, cells were first selected for G418-resistant growth in the presence of IL-7 and 500 μg of Geneticin (Gibco) per ml. When a uniformly viable culture was obtained, the cells were tested for IL-7-independent growth.

To test for tumor formation, 5×10^6 pre-B cells were suspended in 0.3 ml of RPMI 1640 medium lacking serum. The cells were injected into the tail vein (i.v.) or peritoneum (i.p.) of 4- to 6-week-old Bab 14 or C.B.17 SCID mice. Animals were observed over the next 3 months and autopsied when pathology arose. High-molecular-weight DNA was extracted from abnormal tissues as described elsewhere (45). Upon autopsy, spleen, tumor, and thymus weights were noted. Peripheral blood and bone marrow leukocyte counts and differentials were obtained.[2]

RESULTS

Detection of endogenous IL-7 expression in some v-*abl*-transformed pre-B cells. To investigate the possibility that autocrine growth stimulation occurs in transformed pre-B

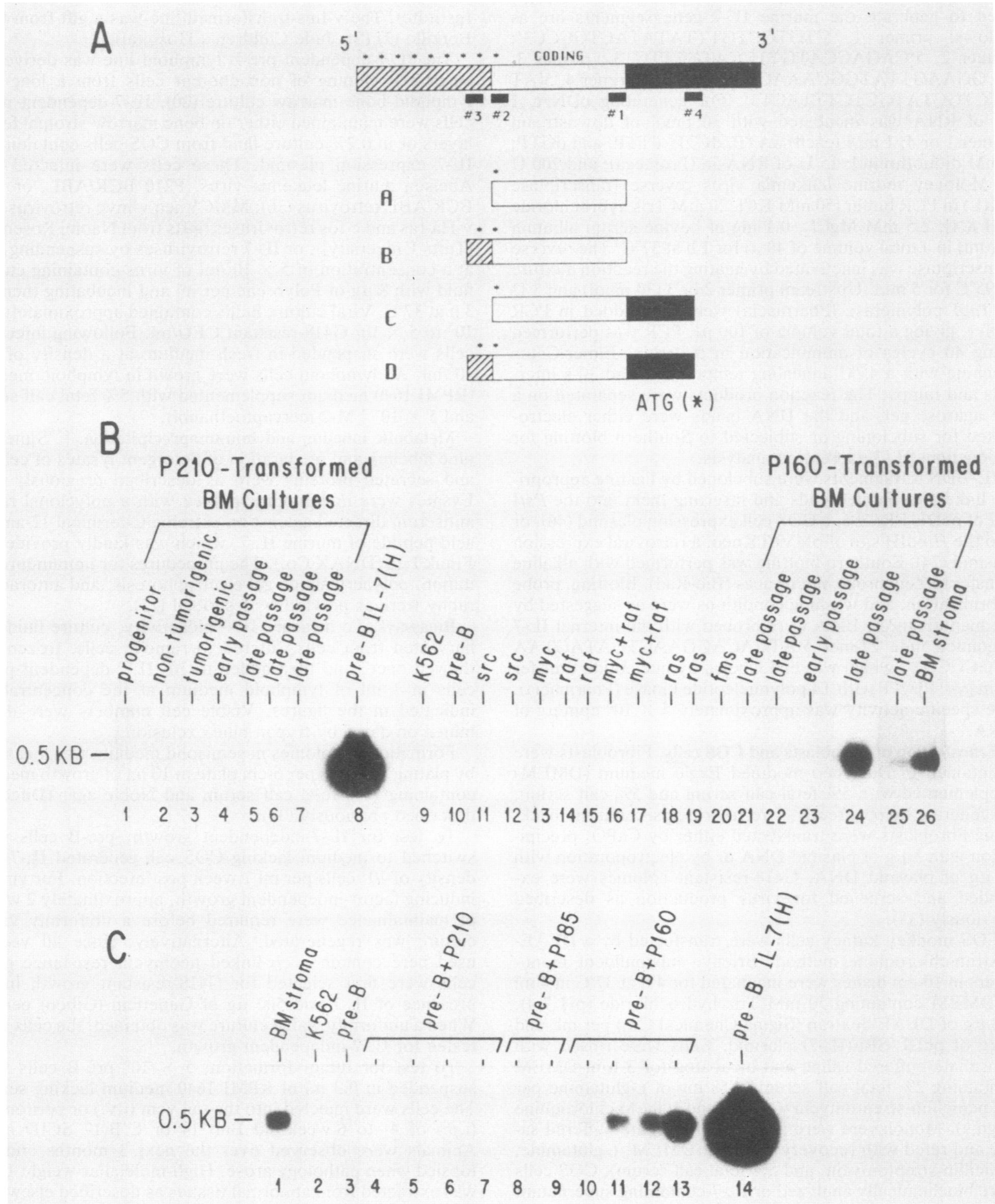

FIG. 1. Detection of IL-7-specific transcripts in transformed pre-B cells by PCR. (A) Schematic drawing of the murine IL-7 cDNA (40) showing the coding region (▭), the 5′ nontranslated region (▨), and the 3′ nontranslated region (■). The presence of an ATG codon is also indicated (*). The positions of oligonucleotide primers 1 through 4 relative to the transcript are indicated by small black rectangles below the cDNA. Their sequences are given in Materials and Methods. The four PCR products generated with these primers are labeled A through D. (B) Southern blot analysis of PCR products amplified from cDNA made from total cytoplasmic RNA of transformed pre-B bone marrow (BM) cultures. Primers 1 and 2 from panel A were used to generate PCR products. The blot was probed with a ^{32}P-end-labeled internal IL-7 oligonucleotide. The phenotypes and transforming genes of the cultures analyzed are indicated above the figure. (C) Analysis was as described for panel B. Cytoplasmic RNAs were obtained from the pre-B cell line rendered IL-7 independent by the proteins indicated (lanes

cells, we examined cells for endogenous IL-7 transcription by using a sensitive PCR protocol. PCR was chosen because IL-7 is biologically active at extremely low levels and is difficult to detect by standard biochemical analysis. Growth assays for secreted bioactivity are sensitive; however, they would not detect intracellularly retained IL-7, which may also stimulate growth. Growth stimulation has been shown to occur in cells containing v-sis (1) or IL-3 (11) constructs engineered for intracellular retention.

We first screened a panel of bone marrow-derived pre-B cell lines transformed by v-*abl*, P210 *BCR-ABL*, v-*src*, v-*raf*, v-*myc*, v-*myc*–v-*raf*, v-Ha-*ras*, or v-*fms* oncogenes. cDNA prepared from total cytoplasmic RNA of these cell lines was subjected to PCR amplification with IL-7-specific oligonucleotide primers 1 and 2 (Fig. 1A). This resulted in an amplified product of 0.47-kb if IL-7 cDNA was present. The products were analyzed by Southern blotting with an internal IL-7 oligonucleotide probe. Since it is possible that IL-7 expression correlates with aggressiveness of the transformed phenotype, we chose for analysis ABL-transformed populations which varied in degree of malignant progression. We compared a P210 BCR/ABL-transformed culture 1 week after outgrowth occurred (Fig. 1B) with stromal layer-independent lines passaged in vitro for over 3 months as well as a line unable to form tumors in animals with fully tumorigenic lines (35). In addition, we examined a P210-expressing B220-positive B-lymphoid line at a stage of differentiation just prior to immunoglobulin H rearrangement (44). We examined five v-*abl*-transformed bone marrow cultures, one within 1 week after transformed outgrowth and four which had been passaged for over 3 months and were feeder independent. All of the non-ABL-transformed lines were late passage and feeder independent (2, 18, 42).

None of the P210 BCR/ABL transformants contained detectable IL-7 transcripts regardless of their transformed phenotypes (Fig. 1B, lanes 1 through 7). In addition, none of the feeder-independent lines transformed by non-*ABL* oncogenes contained transcripts (lanes 11 through 20). However, two of five v-abl transformants, both late passage, contained significantly elevated levels of IL-7 transcripts (lanes 24 and 25). RNAs were prepared and analyzed at least twice to rule out false-positives.

To extend these observations and to control for passage history, we analyzed a panel of pre-B cell lines derived in parallel by infection of an IL-7-dependent pre-B cell line with P160 v-abl, P210 BCR/ABL, P185 BCR/ABL, v-myc, activated c-fos, or v-Ha-ras transforming viruses. By selecting pools of 10^4 infected cells for IL-7-independent growth, clonal outgrowths expressing the ABL transforming proteins P160 v-abl, P210 BCR/ABL, and P185 BCR/ABL were obtained from approximately 75% of the pools. ABL expression was determined by Western blotting (immunoblotting) and immunohistochemical staining (data not shown). This indicates that ABL transforming proteins can bypass or turn on an IL-7 signal within this pre-B cell line. Pre-B cells containing v-myc, c-fos, and v-Ha-ras retroviruses could be obtained by selecting cells infected with these viruses for G418 resistance, since the retroviral vectors also carry the neomycin phosphotransferase gene. However, these cells did not maintain viability without exogenous IL-7, indicating that myc, fos, and ras oncoproteins, unlike activated ABL proteins, cannot abrogate IL-7 dependence in this line.

When cytoplasmic RNAs prepared from the IL-7-independent ABL populations were analyzed by using the PCR amplification assay, none of the BCR/ABL-expressing cells (Fig. 1C, lanes 4 through 9) showed IL-7 transcript levels above those of uninfected cells (Fig. 1C, lane 3). In contrast, the four P160v-abl-expressing populations analyzed contained various but elevated transcript levels (Fig. 1C, lanes 10 through 13). As positive controls for IL-7 transcription we used bone marrow stromal cells which secrete IL-7 and can support the growth of IL-7-dependent cells (Fig. 1B, lane 26, and C, lane 1) and pre-B cells containing an IL-7 expression vector (Fig. 1B, lane 8, and C, lane 14). As a negative control we used the human erythroleukemia line K562, since the human IL-7 gene in not homologous with the murine oligonucleotide primers (Fig. 1B, lane 9, and C, lane 2). Base-line IL-7 transcript levels in the uninfected pre-B cell line are shown in Fig. 1B, lane 10, and C, lane 3.

We conclude that the oncogenic *ABL* genes can induce IL-7-independent growth in pre-B cells. In BCR/ABL transformants this appears to result from a bypass mechanism rather than from autocrine stimulation; therefore, autocrine IL-7 stimulation is not a necessary step in the transformation of pre-B cells. In contrast, despite the similarities in structure and activity between BCR/ABL and v-abl, some v-abl transformants exhibited elevated IL-7 expression.

Strategy for IL-7 overexpression: translation level is augmented by removal of 5′ flanking sequences. It was previously reported that the 5′ flanking region of murine IL-7 cDNA is inhibitory to expression (38). There are eight ATG codons within the first 500 bp upstream of the start codon. To obtain different levels of IL-7, we designed expression constructs which contained the coding region only or included various portions of the 5′ and 3′ flanking regions. Four IL-7 gene segments (A through D) were synthesized from murine primary bone marrow stromal-cell cDNA by PCR with the four primers shown in Fig. 1A. The 5′ flanking region included in segments B and D extends 52 bp upstream of the start codon and contains the most proximal of the upstream ATGs; the 3′ flanking region included in segments C and D extends 250 bp beyond the stop codon.

To determine the regulation imposed by these flanking regions, COS cells were transfected with the pcDL-SRα296 vector containing each of the four IL-7 gene segments and evaluated 3 days later for IL-7-specific DNA, RNA, protein, and secreted bioactivity. Southern blotting of Hirt supernatant DNA and Northern (RNA) blotting of total cytoplasmic RNA showed that plasmid and transcript levels were similar for each of the four constructs (data not shown). However, immunoprecipitations from [^{35}S]methionine-labeled cells or culture fluid showed that both the cell-associated (Fig. 2A, lanes 1 through 4) and the secreted (Fig. 2A, lanes 5 through 8) IL-7 levels produced by the four constructs varied according to the flanking sequences present. Inclusion of 5′ sequences containing one upstream ATG (B and D) caused a 10- to 20-fold reduction in IL-7 protein production, but the 3′ flanking region had no effect. Similarly, the ability of culture

4 through 14). Bone marrow stroma (B, lane 26, and C, lane 1) and pre-B cells expressing the IL-7 (H) construct (B, lane 8, and C, lane 14) were used as positive controls. K562, a human erythroleukemia cell line (B, lane 9, and C, lane 2), and uninfected pre-B cells (B, lane 10, and C, lane 3) were used as negative controls. Exposure was for 15 h at −70°C. Amplification conditions were chosen for maximum detection sensitivity; therefore differences between positive signals may not reflect actual differences in amounts of IL-7 transcripts.

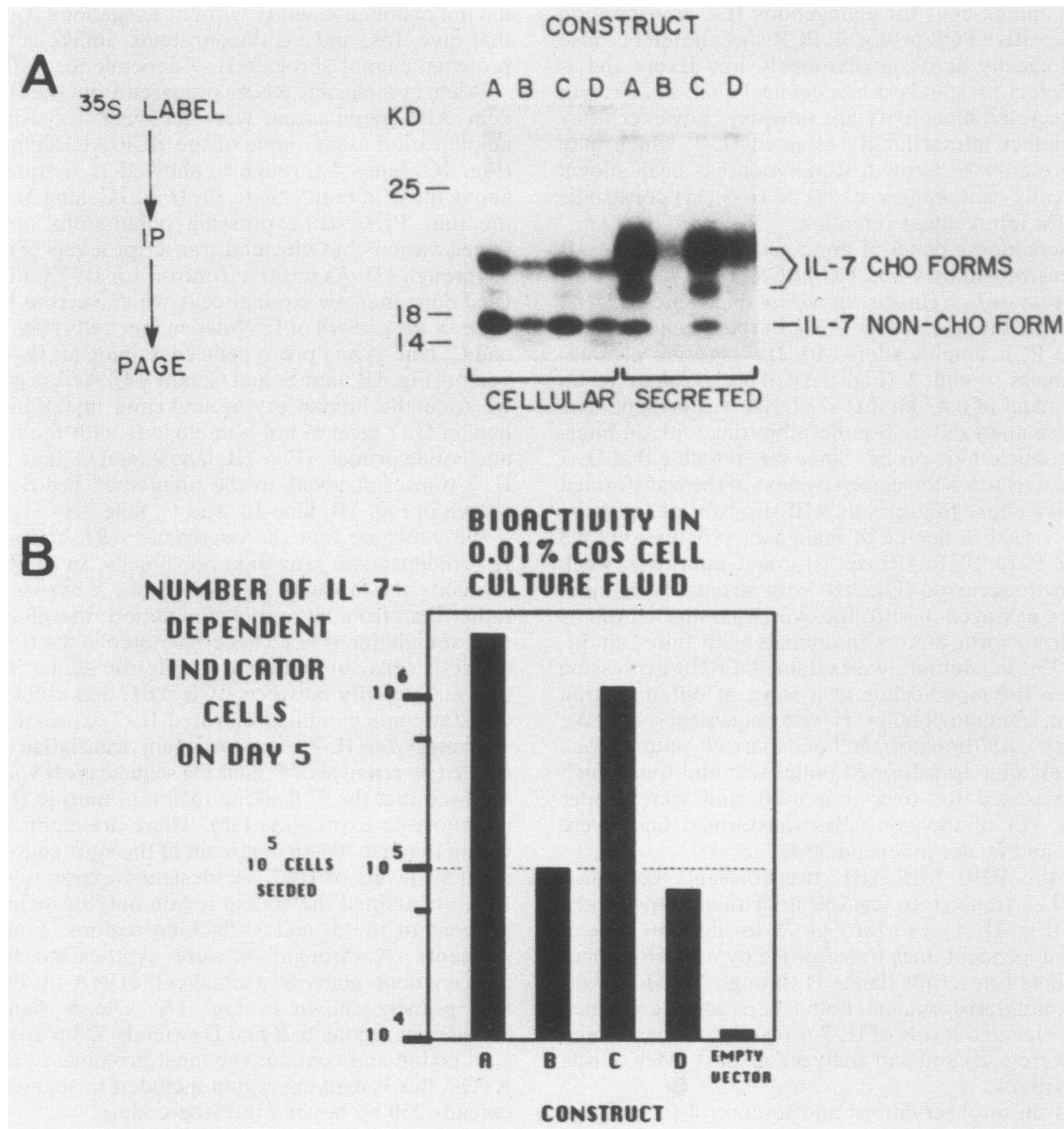

FIG. 2. Translational suppression by IL-7 gene 5′ flanking region in COS cells. (A) COS cells were transfected with pcDL-SRα296 containing the IL-7 gene inserts A through D shown in Fig. 1A. Lysates of [^{35}S]methionine-labeled cellular or secreted proteins from transfected COS cells were immunoprecipitated with anti-IL-7 serum, and the products were resolved on a 12% polyacrylamide gel. The gel was treated for 1 h in 1 M sodium salicylate to enhance the signal. Fluorography was for 24 h at −70°C. Molecular weight markers are shown to the left in kilodaltons. Nonglycosylated and glycosylated forms of IL-7 protein are indicated. (B) Culture fluid in contact with COS cells for 24 h (referred to as conditioned medium) was collected 3 days posttransfection and added at a concentration of 0.01% to 10^5 IL-7-dependent pre-B cells in 1 ml. The number of viable pre-B cells was determined on day 5 by trypan blue exclusion and is indicated by the heights of the bars. Conditioned medium was harvested from COS cells harboring vectors with IL-7 inserts A through D. Empty vector indicates that conditioned medium was harvested from COS cells transfected with a vector carrying no insert.

fluid from the COS cells to stimulate the growth of IL-7-dependent pre-B cells correlated with the levels of IL-7 protein recovered by immunoprecipitation (Fig. 2B). Constructs lacking 5′ flanking sequences directed secretion of at least 10-fold-more IL-7 bioactivity from COS cells. We conclude that IL-7 gene expression is down regulated at the level of translation by the 5′ flanking region and that this regulation can be abolished by removal of these sequences.

Stable expression of IL-7 at two different levels from retroviral vectors. We prepared retroviral vectors which directed two different IL-7 expression levels by cloning segments A or D (Fig. 1A) into the retroviral vector pMV6TKneo. The two resulting constructs, designated IL-7 (H) (high) and IL-7 (SH) (superhigh), were transfected onto the psi-2 fibroblast-packaging line, and helper-free IL-7 retrovirus was collected from G418-resistant clones. Virus-containing culture supernatants were used to infect the IL-7-dependent pre-B cell line. To verify that transfected or infected cells stably contained and expressed the constructs, G418-resistant psi-2 fibroblasts and pre-B cells were analyzed for IL-7-specific DNA, RNA, protein, and secreted bioactivity. Figure 3A shows a comparison by DNA (blot 1) and RNA (blot 2) blot

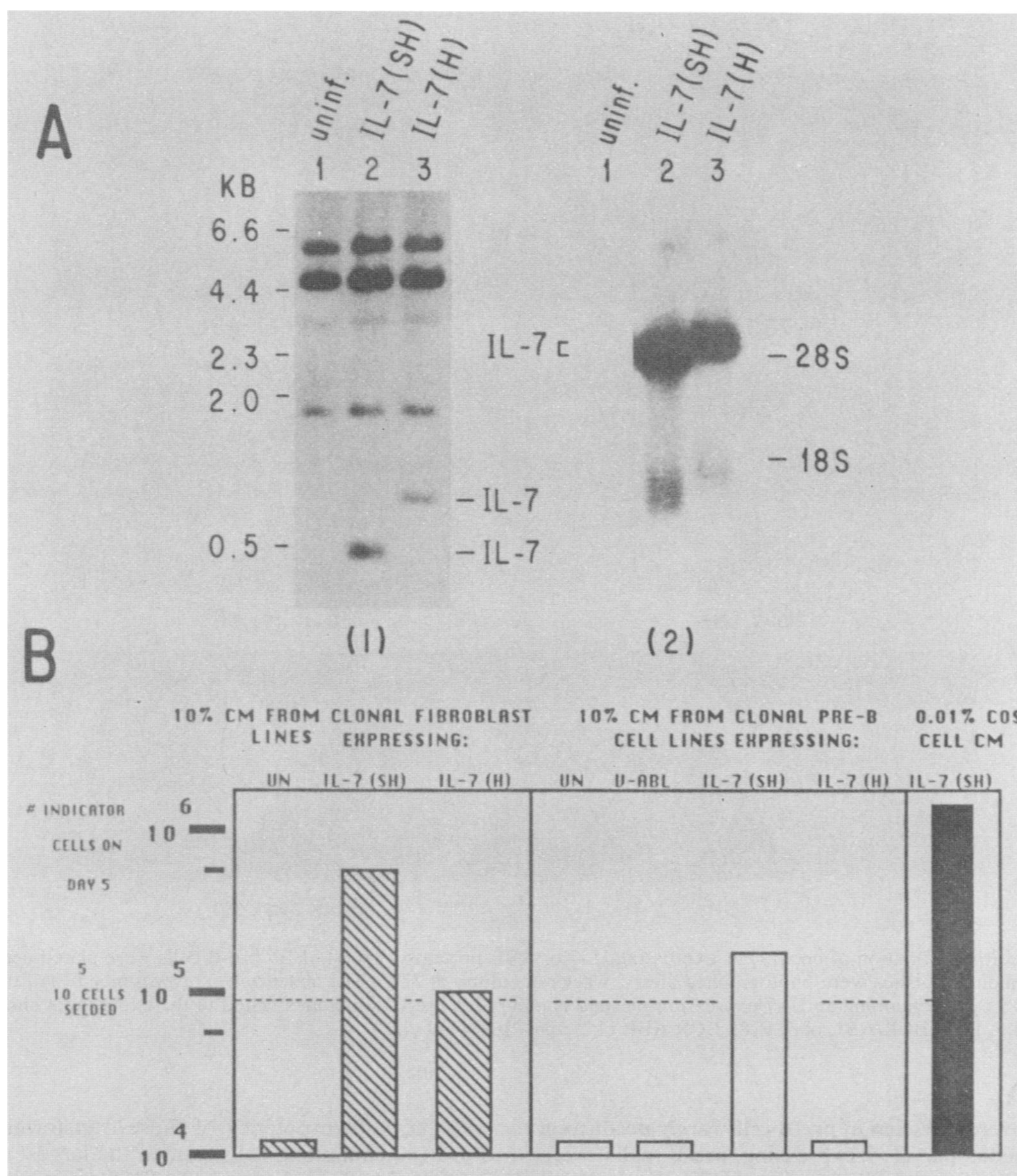

FIG. 3. IL-7 translational level is regulated by flanking sequences when stably expressed from a retroviral vector. (A) IL-7-specific DNA (blot 1) and RNA (blot 2) blot analysis of uninfected (lane 1), IL-7 (H) cl.1 (lane 2), and IL-7 (SH) cl.7 (lane 3) pre-B cells. Nucleic acids were transferred to nitrocellulose membranes and hybridized with a 0.47-kb murine IL-7 gene fragment which was ^{32}P labeled with a random priming kit (BRL). (Blot 1) High-molecular-weight DNA (10 μg) prepared as described previously (48) was cleaved with *Hin*dIII to release the IL-7 insert, and fragments were fractionated on a 1% agarose gel. Size markers are from bacteriophage lambda DNA digested with *Hin*dIII. The 0.47- and 0.7-kb inserts are indicated. Exposure was for 24 h at −70°C. (Blot 2) Total cytoplasmic RNA (20 μg) prepared by urea lysis as described previously (48) was separated on a formaldehyde–1% agarose gel. The migration positions of the 28S (5.23-kb) and 18S (2.15-kb) rRNAs are indicated. Exposure was for 12 h at −70°C. (B) Secreted IL-7 bioactivity. Conditioned media from sources indicated above panel sections were harvested and bioassayed as described in the legend to Fig. 2B. The final concentration of the conditioned media added to 10^5 IL-7-dependent pre-B cells was 10% for medium from fibroblasts (▧) and pre-B cells (□) or 0.01% for medium from COS cells (■). One unit of IL-7 bioactivity was defined as one-half the amount that stimulates 10^5 pre-B cells to double every 24 h. Fibroblasts containing the IL-7 (SH) or IL-7 (H) construct produced 5 or 1 U of bioactivity per ml, respectively. Pre-B cells containing the IL-7 (SH) construct produced 1 to 5 U of bioactivity per ml. Conditioned medium from COS cells containing the IL-7 (SH) construct had 20,000 U of bioactivity per ml.

analysis of pre-B cells harboring the IL-7 (H) or IL-7 (SH) constructs (fibroblast analysis not shown). As was seen in the transient COS expression system, cells containing either construct made similar amounts of IL-7 transcripts. As shown in Fig. 3B, fibroblasts containing the construct without flanking sequences [IL-7 (SH)] secreted fivefold-more IL-7 bioactivity than cells containing the construct with flanking sequences [IL-7 (H)]. No bioactivity could be detected in the supernatant of the pre-B cells containing the IL-7 (H) construct. Secreted activity varied among individually isolated pre-B lines containing the IL-7 (SH) construct, and an average value is shown in Fig. 3B.

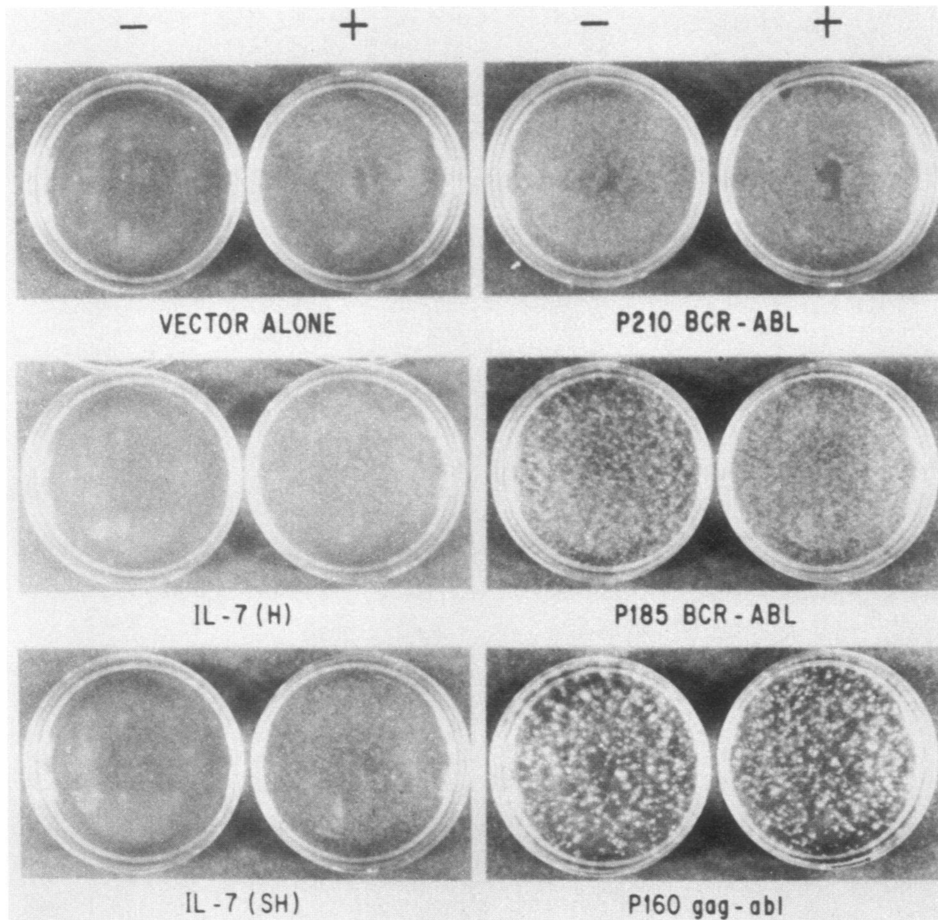

FIG. 4. Agar colony formation of pre-B cells expressing IL-7 or ABL proteins. A total of 10^5 pre-B cells were plated per 6-cm dish in 10 ml of semisolid medium. Dishes were photographed after 2 weeks of culture at 37°C. The absence (−) or presence (+) of 0.2% conditioned medium from COS cells containing an IL-7 expression plasmid is indicated. Pre-B-cell clones tested in the experiment shown are IL-7 (H) cl.1, IL-7 (SH) cl.5, P210 BCR/ABL cl.3, P185 BCR/ABL cl.2, and P160 v-abl cl.2.

Forced IL-7 overexpression in pre-B cells rarely produces a tumorigenic phenotype. IL-7-expressing pre-B cells were examined for transformed characteristics. To determine if IL-7 produced internally allows autocrine growth, we tested pre-B IL-7 (H) and IL-7 (SH) cells for their abilities to grow in the absence of exogenously added IL-7. Uninfected pre-B cells in growth medium containing excess COS cell-produced IL-7 (0.1% culture fluid or 20 U/ml) grow with a maximum doubling time of 24 h. When IL-7 is removed, all cells die within 48 h. Pre-B cells expressing the IL-7 (H) construct remained viable in the absence of exogenous IL-7 and grew with a doubling time of approximately 48 h, whereas cells expressing the IL-7 (SH) construct doubled every 16 to 24 h, independent of exogenous IL-7.

The ability to form colonies in semisolid medium and tumors in syngeneic animals are two stringent tests for the transformed phenotype. Clonal populations of pre-B cells containing IL-7 (H) or IL-7 (SH) constructs were compared with uninfected pre-B cells and with cells containing P210 BCR/ABL, P185 BCR/ABL, or P160v-abl, which were described above (Fig. 1C). Cells expressing each of the three activated forms of ABL could readily form colonies in agar medium regardless of whether exogenous IL-7 was included in the medium (Fig. 4). The sizes of the colonies correlated with the known potency of these transforming agents (32, 36): P160-containing cells formed the largest colonies, ranging from 1 to 3 mm in diameter; P185-containing cells formed intermediate-sized colonies of approximately 1 mm; and P210-containing cells produced colonies of approximately 0.5 mm. In contrast, cells harboring either the IL-7 (H) or IL-7 (SH) constructs displayed a colony phenotype indistinguishable from that of uninfected cells. They could form numerous pinpoint colonies in the presence of exogenous IL-7 (Fig. 4) but in its absence remained single cells as determined by microscopic examination.

To test for tumorigenic potential, we used both immunocompetent Bab 14 mice and immunodefective SCID mice as recipients. Bab 14 mice are genetically identical to the pre-B cell line except for a substitution of sequences in the immunoglobulin H locus which creates a diagnostic restriction fragment length polymorphism (39). SCID mice lack functional T or B lymphocytes and are compromised in their abilities to reject allografts or even xenografts (4). Cells (5×10^6) were injected i.v. or i.p. into 4- to 6-week-old mice. The frequency and latency of tumor formation are shown in Table 1. Uninfected pre-B cells or those infected with vector alone produced no pathology in Bab 14 mice even after 3 months. In SCID mice, a mild degree of mesenteric lymph

TABLE 1. Tumorigenic potential of pre-B cells expressing IL-7 or ABL proteins

Virus[a]	Clone	Route	Strain	Tumor frequency[b]	Latency (wk)[c]	
					Avg	Range
None		i.v.	Bab 14	0/4		
		i.p.	Bab 14	0/3		
Vector alone		i.v.	Bab 14	0/2		
		i.p.	Bab 14	0/3		
		i.p.	SCID	0/3		
IL-7 (H)	cl.1	i.v.	Bab 14	0/4		
		i.p.	Bab 14	0/6		
		i.p.	SCID	0/3		
IL-7 (SH)	cl.1	i.p.	Bab 14	0/3		
	cl.3	i.p.	Bab 14	0/3		
	cl.4	i.p.	Bab 14	0/3		
	cl.5	i.v.	Bab 14	4/4	7	6–8
		i.p.	Bab 14	3/3	5	4–6
		i.p.	SCID	3/3	5	4–6
	cl.6	i.p.	Bab 14	0/3		
	cl.7	i.p.	Bab 14	0/3		
P210 BCR/ABL	cl.1	i.v.	Bab 14	3/4	6	5–7
	cl.3	i.v.	Bab 14	4/4	6	5–7
		i.p.	SCID	3/3	5	4–6
P185 BCR/ABL	cl.2	i.v.	Bab 14	4/4	4	4
P160v-abl	cl.2	i.v.	Bab 14	4/4	3.5	3–4
	cl.6	i.p.	SCID	3/3	4	4

[a] Pre-B cell lines expressing the constructs shown were injected i.v. or i.p. into 4- to 6-week-old Bab 14 or SCID mice. Each mouse received 5×10^6 cells suspended in 0.3 ml of RPMI 1640 medium. Mice were observed for 3 months.

[b] Number of mice with tumors/number of mice tested.

[c] Time postinjection when pathology was first observed.

node enlargement was observed in approximately half of the animals upon autopsy 3 months postinjection. These animals otherwise appeared healthy. In contrast, tumors formed rapidly in every animal injected with pre-B cells expressing oncogenic ABL proteins (Table 1).

The IL-7 (H)-expressing cells behaved essentially as control cells, producing no pathology other than slight mesenteric lymph node enlargement in the SCID mice. One IL-7 (SH)-expressing clone, cl.5, produced tumors in every animal injected (a total of 10), with latencies similar to those of P210-expressing cells. However, five other independently derived IL-7 (SH) lines with comparable IL-7 secretion levels produced no pathology after 3 months. At autopsy all tumor-bearing animals had enlarged spleens and lymph nodes, elevated leukocyte counts, and a predominance of mononuclear cells in the bone marrow and peripheral blood. Mice injected i.p. frequently had tumors at the injection site.

Genomic DNAs prepared from the donor cell lines and tumor tissues were compared by Southern blotting for the presence of the construct insert and proviral integration site. The tumor tissues contained the ABL or IL-7 construct inserts and the same clonal integration sites as the donor cell lines (data not shown).

DISCUSSION

A substantial body of evidence exists for involvement of deregulated growth factor pathways in malignancy. Several retroviral oncogenes have been found to encode activated forms of growth factors (platelet-derived growth factor and fibroblast growth factor) or growth factor receptors (epidermal growth factor receptor, colony-stimulating factor receptor [CSF-1-R], and c-kit). The recent finding that the env protein of the Friend spleen focus-forming virus mimics erythropoietin by binding and activating its receptor is a novel example (30).

The IL-7 gene could serve as a target for mutational activation. A simple mechanism for deregulation of IL-7 expression is suggested by its remarkable 5′ nontranslated sequence, which contains eight ATG codons within the first proximal 500 bp (33). Others have shown previously (38) and we show in this report that removal of 5′ nontranslated sequence dramatically elevates the IL-7 protein level. Mutation or rearrangement of this region could alter IL-7 translational levels significantly, as was shown to occur in *lck* gene expression in LSTRA cells (13).

Overexpression of normal growth factors in hematopoietic cells via retrovirally mediated gene transfer has demonstrated the potential of these factors to act as cofactors in leukemogenesis. IL-3 (22, 50) or granulocyte-macrophage CSF (GM-CSF) (27) expression in established factor-dependent lines can cause conversion to a tumorigenic phenotype. In contrast, expression in primary cells has not resulted in malignancy. Reconstitution of mice with bone marrow infected with an IL-3 retrovirus (5, 49) or GM-CSF retrovirus (23) or creation of mice transgenic for a deregulated GM-CSF gene (28) resulted in nonneoplastic myeloproliferative diseases. Hyperexpression of normal growth factors may contribute to leukemogenesis, but synergy with secondary events is generally required.

Our pre-B cell line containing the IL-7 (H) construct retaining one upstream ATG codon could grow in the absence of exogenous IL-7 but did not secrete appreciable IL-7 bioactivity. This suggests that sufficient IL-7 stimulation to prevent apoptosis and maintain nonneoplastic growth may result from binding of IL-7 to receptors intracellularly. Cells containing the IL-7 (SH) construct, which lacks all flanking sequences, secreted IL-7 in addition to attaining autocrine growth. Despite these properties, none of the cell lines displayed an augmented colony-forming ability on agar, and only one of the six IL-7 (SH) lines tested was tumorigenic. Our results resemble those obtained for CSF-1 expression in established cells in which factor-independent growth without tumorigenesis was observed (43).

A novel and unexpected finding in this study is the induction of endogenous IL-7 transcription in v-abl- but not in BCR/ABL-transformed pre-B cells. v-abl and BCR/ABL are structurally and functionally similar. Differences in their transforming activities are for the most part quantitative rather than qualitative. Two structural differences are that v-abl is myristylated while BCR/ABL is not and in v-abl the regulatory src homology 3 (SH3) region (12, 21) has been truncated while in BCR/ABL it is intact. Differences in intracellular location or degree of kinase domain deregulation may result in differences in gene activation induced by these two transforming proteins.

Transformation by activated ABL proteins can produce independence from several growth factors in hematopoietic cells, including IL-2 in T cells (8), IL-3 in myeloid cells (9, 10, 16, 19, 41), and IL-7 in pre-B cells. Both autocrine and bypass mechanisms have been proposed to explain growth factor independence and transformation (9, 10, 16, 19, 41). We have observed that among a series of ABL-transformed IL-7-independent pre-B cells lines, some do and some do not express IL-7.

We conclude that autocrine expression of IL-7 is not necessary for transformation of pre-B cells in general, since it was absent in pre-B cells transformed by various oncogenes, including some v-abl transformants. The expression

level detected in some v-abl transformants is clearly not sufficient to produce their transformed phenotypes, since forced IL-7 expression in the same parental pre-B cell line could not duplicate this phenotype. Our data are most consistent with IL-7 expression being one of several alternative events whose synergy produces transformation of pre-B cells.

ACKNOWLEDGMENTS

We thank Mike Cohen and Carol Crookshank for excellent photographic and secretarial work on the manuscript. We thank Charles Sawyers, Ann Marie Pendergast, and Arnie Berk for critical reading of the manuscript; Frank Lee and Nick Wrighton (DNAX) for the gift of anti-IL-7 serum and the procedure for transfection of COS cells; and Jacalyn Pierce (National Institutes of Health) and Gary Borzillo (St. Jude) for their gifts of transformed pre-B cell lines.

This work was supported by research grants from the National Cancer Institute to O.N.W. J.C.Y. and M.L.G. are postdoctoral fellows of the Leukemia Society of America. O.N.W. is an investigator of the Howard Hughes Medical Institute.

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