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Subcellular Localization of Bcr, Abl, and Bcr-Abl Proteins in Normal and Leukemic Cells and Correlation of Expression with Myeloid Differentiation

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Abstract

We used specific antisera and immunohistochemical methods to investigate the subcellular localization and expression of Bcr, Abl, and Bcr-Abl proteins in leukemic cell lines and in fresh human leukemic and normal samples at various stages of myeloid differentiation. Earlier studies of the subcellular localization of transfected murine type IV c-Abl protein in fibroblasts have shown that this molecule resides largely in the nucleus, whereas transforming deletion variants are localized exclusively in the cytoplasm. Here, we demonstrate that the murine type IV c-Abl protein is also found in the nucleus when overexpressed in a mouse hematopoietic cell line. However, in both normal and leukemic human hematopoietic cells, c-Abl is discerned predominantly in the cytoplasm, with nuclear staining present, albeit at a lower level. In contrast, normal endogenous Bcr protein, as well as the aberrant p210^{BCR-ABL} and p190^{BCR-ABL} proteins consistently localize to the cytoplasm in both cell lines and fresh cells. The results with p210^{BCR-ABL} were confirmed in a unique Ph¹-positive chronic myelogenous leukemia (CML) cell line, KBM5, which lacks the normal chromosome 9 and hence the normal c-Abl product. Because the p210^{BCR-ABL} protein appears cytoplasmic in both chronic phase and blast crisis CML cells, as does the p190^{BCR-ABL} in Ph¹-positive acute leukemia, a change in subcellular location of Bcr-Abl proteins between cytoplasm and nucleus cannot explain the different spectrum of leukemias associated with p210 and p190, nor the transition from the chronic to the acute leukemia phenotype seen in CML.

Further analysis of fresh CML and normal hematopoietic bone marrow cells reveals that p210^{BCR-ABL}, as well as the normal Bcr and Abl proteins, are expressed primarily in the early stages of myeloid maturation, and that levels of expression are reduced significantly as the cells mature to polymorphonuclear leukocytes. Similarly, a decrease in Bcr and Abl levels occurs in HL-60 cells induced by DMSO to undergo granulocytic differentiation. The action of p210^{BCR-ABL} and its normal counterparts may, therefore, take place during the earlier stages of myeloid development. (*J. Clin. Invest.* 1993. 92:1925-1939.)
Key words: chronic myelogenous leukemia • hematopoiesis •

Bcr-Abl fusion proteins • c-ABL protooncogene proteins • immunohistochemistry

Introduction

Clonal expansion of a hematopoietic stem cell containing a reciprocal translocation between chromosomes 9 and 22 (the Philadelphia translocation [Ph]¹) characterizes chronic myelogenous leukemia (CML) and subsets of acute leukemia (1, 2). This translocation results in the head-to-tail fusion of variable numbers of 5' exons of the *BCR* gene on chromosome 22 with the *c-ABL* gene derived from chromosome 9 (3-5). The resultant chimeric gene is transcribed into a hybrid *BCR-ABL* mRNA in which exon 1 of *c-ABL* is replaced by 5' *BCR* exons. Bcr-Abl fusion proteins (p210^{BCR-ABL} and p190^{BCR-ABL}) are produced; they contain the NH₂-terminal 927 (or 902) or 426 amino acids of Bcr and are implicated in the development of CML and Ph¹-positive acute leukemia, respectively (3, 6-9).

The oncogenic potential of the Bcr-Abl fusion proteins has been validated by their ability to transform hemopoietic progenitor cells in vitro (10-14). Furthermore, transgenic mice carrying *BCR-ABL* constructs develop lymphoid tumors (15, 16), and reconstituting lethally irradiated mice with bone marrow cells infected with retrovirus carrying the gene encoding p210^{BCR-ABL} leads to the development of several fatal hematopoietic neoplasms (17-19). By exploiting different retroviral constructs, a myeloproliferative syndrome resembling CML can be induced in 50% of the experimental animals; the remaining animals succumb to pre-B leukemia/lymphoma, or tumors of erythroid and macrophage cell types. Taken together, these data provide cogent evidence for *BCR-ABL* gene participation in leukemogenesis.

Unfortunately, there still remain significant gaps in our understanding of the role of the normal Abl and Bcr proteins, as well as the aberrant p210^{BCR-ABL} and p190^{BCR-ABL} in human hematopoiesis. In this regard, two types of information might be relevant. First, the types of cells that express a particular protein might be informative. Yet, despite the presumed role of Bcr, Abl, and Bcr-Abl in hematopoiesis, little is known about when these proteins are expressed during hematopoietic development. Second, subcellular localization of a protein often provides significant clues to its function, and the transition from the benign state to the fully malignant one might involve a change in subcellular residence. Importantly, such an event has been inferred from experiments with transfected mouse type IV *c-ABL*. The mammalian *c-ABL* gene produces two proteins of 145 kD that differ only at their NH₂ terminus, denoted types I and IV c-Abl in mice, and types Ia and Ib c-Abl in

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1. Abbreviations used in this paper: ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia, Ph¹, Philadelphia translocation.

humans, respectively. As demonstrated by immunohistochemistry, the protein product of normal mouse type IV *c-ABL* constructs transfected into fibroblasts resides mostly in the nucleus whereas constructs with a small NH₂-terminal deletion produce a protein that has been moved to the cytoplasm and whose transforming potential has been fully activated (20). In this study, we have assessed the subcellular localization and differential expression of Bcr, Abl, and Bcr-Abl proteins in Ph¹-positive CML and acute lymphoblastic leukemia (ALL) cell lines, as well as in fresh leukemic and normal samples. We have also exploited a unique p210^{BCR-ABL}-positive CML line (KBM-5), which lacks the normal chromosome 9 and, hence, the normal *c-ABL* product. Our observations suggest that normal endogenous Bcr protein, as well as the aberrant p210^{BCR-ABL} and p190^{BCR-ABL} localize to the cytoplasm in both cell lines and fresh cells. While the mouse type IV *c-Abl* protein resides mostly in the nucleus in hematopoietic cells and in fibroblasts, the majority of endogenous human *c-Abl* protein is found in the cytoplasm of hematopoietic cells and cell lines, though some nuclear staining is also present. In addition, a marked decrease in expression of Bcr and *c-Abl* is seen in the HL-60 leukemia cell line after induction of granulocytic differentiation. Analysis of fresh CML and normal hematopoietic cells reveals that p210^{BCR-ABL} and the normal Bcr and Abl proteins are also expressed primarily in the early stages of myeloid maturation, and that levels of expression are reduced significantly as the cells differentiate.

Methods

Cell lines. We used the CML blastic crisis cell line K562 (Ph¹-positive), the promyelocytic leukemia cell line HL-60 (Ph¹-negative), the acute myelogenous leukemia cell line KG-1 (Ph¹-negative) (all obtained from American Type Culture Collection, Rockville, MD), the Ph¹-positive acute lymphoblastic leukemia cell line ALL-1 (kindly provided by Dr. G. Rovera, Wistar Institute, Philadelphia, PA), a recently established Ph¹-positive CML cell line designated KBM-5 (Beran, M., unpublished data) and Ba/F3 cells (21), a B-lymphoid line that overexpresses the myristoylated form of mouse *c-Abl* (*c-Abl* type IV) (22) (generously provided by Dr. George Daley, Massachusetts General Hospital, Boston, MA). All cell lines except KBM-5 were cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) containing 10% inactivated fetal calf serum (Hyclone Laboratories, Logan, UT) in a 5% CO₂ incubator at 37°C; the medium for Ba/F3 cells was supplemented with 5% WEHI-3B conditioned medium as a source of interleukin-3. KBM-5 cells were grown in Iscove's medium (GIBCO BRL, Grand Island, NY) with 15% inactivated fetal calf serum.

Northern analysis. To evaluate the presence of *BCR*, *ABL*, and *BCR-ABL* messages, total RNA was prepared as previously described (23). Poly(A)⁺ selected mRNA (4 µg/lane) was then size-separated in 1.1% agarose/formaldehyde gels, transferred to nitrocellulose filters, and hybridized to ³²P-labeled cDNA probes. Preparation of cDNA probes and hybridization conditions used for the Northern analysis have been previously described (24). The *BCR* probe was derived from the 0.45 kb EcoR1/PstI cDNA fragment located at the 5' end of clone K28. This fragment is composed entirely of non-*ABL* sequences (25). The *c-ABL* probe was derived from an EcoR1/BamHI cDNA fragment corresponding to the most 5' *v-ABL* hybridizing region (26).

Southern analysis. To evaluate the configuration of the *BCR* gene, DNA was prepared, as previously described (27), and 10 µg of DNA was digested with BamHI and BglII restriction endonucleases in conditions recommended by the supplier of the endonucleases (International Biotechnologies, Inc., New Haven, CT), electrophoresed in 0.8% agarose gel, blotted, and hybridized according to the method of Southern (28). The universal *BCR* probe encompassing most of the

5.8 kb *BCR* region (Ph1/bcr-3) (Oncogene Science, Inc., Manhasset, NY) was labeled by oligoprimers extension to a specific activity of 1–3 × 10⁹ cpm/µg of DNA (29). After hybridization, the filters were washed at 60°C for 1 h in 0.1 × SSC solution (SSC = 0.15 mol/liter sodium chloride, 0.015 mol/liter sodium citrate) containing 0.1% sodium dodecyl sulfate, dried, and autoradiographed.

PCR. Because cytogenetic analysis suggests that KBM-5 cells have lost their normal chromosome 9, we performed PCR to evaluate the presence of normal *ABL* message in these cells. 1 µg of total RNA was used for reverse transcription to cDNA and amplification reactions, as previously described (30), and the amplification was performed for 40 cycles. Detection was performed using the hybridization protection assay, according to methodology identical to that previously used in our laboratory (31). The 5' primer was a 20-mer beginning 65 bases upstream of the *c-ABL* exon Ib/II junction. The 3' primer used is as previously described (30). An acridinium ester-labeled oligonucleotide complementary to *ABL* exon Ib/II junction sequences was synthesized by Gen-Probe (San Diego, CA) (32). This probe is a 24-mer spanning the splice junction with 14 bases in exon II.

Similarly, to determine the presence of normal *BCR* message in KBM-5 cells, we amplified normal *BCR* cDNA using the following primers: *BCRa* 5'-AAGGCAGCCTTCGACGTCATAAC-3', *BCRb* 5'-GACTTCGGTGGAGAACAGGATGCT-3'. Southern blotting and hybridization with an oligonucleotide probe 5'-ATTGACTGCCCTCCTTCTCTGCCAC-3' was done for detection purposes according to the method of Kawasaki and colleagues (30).

Immune complex kinase assay. p210^{BCR-ABL} and p190^{BCR-ABL} were detected by exploiting their tyrosine phosphokinase enzymatic activity in the immune complex kinase assay (7). The antiserum used for this assay was anti-Abl389-403, a rabbit polyclonal serum made against the predicted hydrophilic domains of *v-Abl* (7). The immune complex kinase assay was performed on 2 × 10⁷ cells. To ensure that the 210 and 190 bands on the gels represented proteins recognized by the anti-Abl serum, rather than background phosphorylation, alternate samples were incubated with anti-Abl serum and blocking cognate peptide (7, 33).

Fresh cells. Peripheral blood was obtained from five CML Ph¹ patients in the chronic phase of the disease, five patients at blast crisis, two individuals with acute myelogenous leukemia (Ph¹-negative), and three normal volunteers. In addition, two bone marrow samples derived from normal donors when marrow was collected for allogeneic transplant served as normal controls. Sample collections were performed at M. D. Anderson Cancer Center (Houston, TX) in accordance with institutional guidelines, and after obtaining informed consent from all participants. Red blood cells were lysed with 1.22% ammonium oxalate (Sigma Immunochemicals, St. Louis, MO), and the white blood cells were washed with PBS. To obtain blasts and granulocytes for immunoblotting, cells from a CML myeloid blast crisis patient were separated on Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) discontinuous gradient, and fractions 1.045 and 1.080 g/ml were collected.

Immune reagents for immunofluorescence and immunohistochemistry. For detection of Abl protein, rabbit polyclonal antisera against *v-Abl* trpE bacterial fusion protein pEX4 (kindly provided by Dr. Owen Witte, Howard Hughes Medical Institute, University of California at Los Angeles) was used (33). Soluble PEX4 protein for affinity purification of antisera and blocking purposes was obtained by expressing the PEX4 sequence as part of a bacterial fusion protein, with the *Schistosoma japonicum* glutathione S-transferase protein in the glutathione expression system, as previously described (20, 34). For detection of Bcr protein, two antisera were used: (a) rabbit polyclonal sera against β-galactosidase/Bcr fusion protein (35), which was affinity-purified using glutathione S-transferase Bcr fusion protein; and (b) monoclonal antibody against Bcr (7C6), originally described by Dhut et al. (36) (Oncogene Science, Inc. Uniondale, NY). All these antisera have been extensively characterized (33–36).

Immunofluorescence. Cells were cytopun on glass slides at a density of 1 × 10⁵ cells/slide. All subsequent steps were carried out at room

temperature. Slides were fixed in 4% paraformaldehyde (Sigma Immunochemicals) in PBS for 10 min. Paraformaldehyde fixed cells were quenched in 0.1 M 2-ethanolamine pH 8.0 (Sigma Immunochemicals) for 5 min, then permeabilized by 0.2% Triton X-100 (Bio-Rad, Richmond, CA) in PBS for 10 min. Cells were then blocked with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. Primary antibodies were used at a 5–20 $\mu\text{g}/\text{ml}$ concentration. Where indicated, affinity purified anti-pEX4 antiserum was blocked with pEX4 fusion protein by incubation with a 10-fold molar excess of purified protein for 30 min before application to cells. In all experiments, normal rabbit IgG (Jackson ImmunoResearch Laboratories) was used as a negative control for anti-pEX4 sera and for the rabbit polyclonal anti-Bcr sera. Isotypic antibody was used as a control for the monoclonal anti-Bcr (7C6) sera. Incubation of primary antibody was for 45 min, followed by extensive washing in PBS. The appropriate secondary antibody (Jackson ImmunoResearch Laboratories) was added at a 1:100 dilution for 30 min, after which the cells were washed briefly with PBS and mounted with *N*-propyl gallate (Sigma Immunochemicals). Conventional epifluorescence microscopy was performed with an Olympus Vanox AH-2 fluorescence microscope. Photography of fluorescence specimens was accomplished with Kodak Tmax ASA 400 black and white 35-mm film and Kodak Ektachrome ASA 800/1600 color film.

Alkaline phosphatase staining. All steps were carried out at room temperature. Slides were fixed and treated as above. Alkaline phosphatase-tagged secondary antibodies (Jackson ImmunoResearch Laboratories) were added at a 1:200–1:300 dilution. The alkaline phosphatase reagents (Dako Corp., Carpinteria, CA) were applied for 10 min, after which the cells were washed briefly with water, counterstained with methyl green (Roboz Surgical Instrument Co., Inc., Washington, D.C.) for Abl and methylene blue (Fisher Scientific, Houston, TX) for Bcr, dehydrated, and mounted with Aqua-Mount (Lerner Laboratories, Pittsburgh, PA) or Permount (Fisher), respectively.

In vitro differentiation induction of HL-60 cells. To further dissect the expression of Bcr and Abl proteins in relation to the stage of differentiation, HL-60 cells were induced to undergo granulocytic differentiation by exposure to 1.25% dimethyl sulfoxide (37) in a 5% CO₂ incubator at 37°C for 5 d. The cells were then cytopun and processed as described above for immunofluorescence reactions.

Western blotting. Immunoblotting was performed to further assess Bcr-Abl steady state protein levels. Briefly, our procedure was as follows: 2×10^7 lyophilized cells were lysed in Western sample buffer (10 mmol/liter Tris-HCL, 1 mmol/l EDTA, 1% SDS, 5% glycerol, 1% β -mercaptoethanol, at final pH of 8.0 containing 0.1% bromo-phenol-blue). Boiled and cleared lysates (12,000 $g \times 30$ min) were fractionated on 8% SDS-polyacrylamide gels. The fractionated proteins were transferred onto 0.1- μm nitrocellulose membrane in 25 mM Tris-HCL, 19 mM glycine, 0.1% SDS plus 20% methanol at 40 V overnight at 4°C. The nitrocellulose blot was incubated with either rabbit anti-v-Abl sera or preimmune sera in TNE/NP-40 solution (50 mmol/liter Tris-HCL, 250 mmol/liter NaCl, 2 mmol/l EDTA, 0.1% Nonidet P-40) containing 3% BSA (fraction 5; Sigma Immunochemicals) overnight. This sera was made by immunizing rabbits with a truncated product, p160^{v-ABL} of the Abelson murine leukemia virus. Complete details of the antisera have been previously published (38). The blot was rinsed with TNE/NP-40 and then incubated with 0.2 $\mu\text{Ci}/\text{lane}$ of ¹²⁵I-protein A (IM144; Amersham Corp., Arlington Heights, IL) in TNE/NP-40 containing 3% BSA at 37°C. After further washing with TNE/NP-40, the blot was autoradiographed using an intensifying screen at –70°C.

Results

Northern blot and polymerase chain reaction analysis of cell line BCR, ABL and BCR-ABL RNA. Hybridization with c-ABL and BCR cDNA probes revealed the characteristic 8.5-kb BCR-ABL mRNA in the two CML cell lines: K562 and KBM-5. The normal 6- and 7-kb c-ABL mRNA species were present

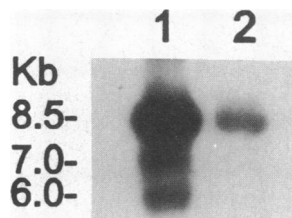


Figure 1. Northern blot of mRNA from K562 cells (lane 1) and KBM-5 cells (lane 2). The blot was hybridized with an EcoRI/BamHI cDNA fragment corresponding to the most 5' v-ABL hybridizing region (26).

in K562 cells (Fig. 1, lane 1) but not in KBM-5 cells (Fig. 1, lane 2). Likewise, PCR analysis followed by HPA did not detect the normal ABL cDNA in KBM-5 cells (data not shown), confirming the absence of these sequences in KBM-5 cells; these data are consistent with the karyotypic analysis of KBM-5 cells, which demonstrates loss of the normal chromosome 9 (39). PCR analysis did detect the normal BCR cDNA sequence in KBM-5 cells (data not shown). (The cell line KBM-5 was previously described to lack the normal BCR gene based on the presence of only a rearranged BCR band [without the germline] on Southern blot of DNA hybridized with a 3' 1.2-kb HindIII/BglII BCR genomic probe [39]. However, by hybridizing a Southern blot containing DNA from KBM-5 cells with the larger [5.8 kb] universal Ph1/bcr-3 probe, we were able to show the presence of the germline BCR DNA. These results suggest that a 3' BCR sequence deletion has occurred in KBM-5 cells, accounting for the previously published results, and consistent with our observation of normal BCR transcripts in this cell line.)

Immune complex kinase analysis of p210^{BCR-ABL} and p190^{BCR-ABL} proteins. The immune complex kinase assay can detect Abl-related proteins because they are enzymatically active as phosphokinases. In this assay, K562 and KBM-5 cells showed p210^{BCR-ABL} whereas ALL-1 cells showed p190^{BCR-ABL}. HL-60 and KG-1 cells showed neither p210^{BCR-ABL} nor p190^{BCR-ABL} (Fig. 2). (The antiserum used for this assay was anti-Abl389-403 [7], and we have previously determined that this antibody is unable to detect the normal c-Abl protein [40, 41].)

p210^{BCR-ABL} and p190^{BCR-ABL} are cytoplasmic in Ph¹-positive leukemic cell lines. The Ph¹-positive human CML and ALL cell lines (K562 and ALL-1) express p210^{BCR-ABL} and p190^{BCR-ABL}, respectively, at levels about 10-fold greater than the normal c-Abl (as determined by Western blotting (Van Etten, R. A. and R. Kurzrock, unpublished data)) so that both anti-Abl and anti-Bcr antibodies predominantly detect Bcr-Abl under the conditions we used (Figs. 3–5). K562 cells showed strong immunoreactions localized to the cytoplasm when the anti-pEX4 sera was used (Fig. 3 A). Cytoplasmic localization of p210^{BCR-ABL} was further confirmed by studying KBM-5 cells (Fig. 5 A) (which lack the normal Abl product) eliminating the possibility of colocalization with the normal counterpart as a

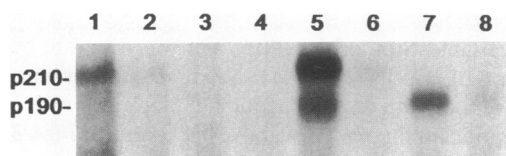


Figure 2. Immune complex kinase assay of KBM-5 cells (lanes 1 and 2), HL-60 cells (lanes 3 and 4), K562 cells (lanes 5 and 6), and ALL-1 cells (lanes 7 and 8). Antisera used was the anti Abl 389-403 (7). Even lanes were blocked with cognate peptide.

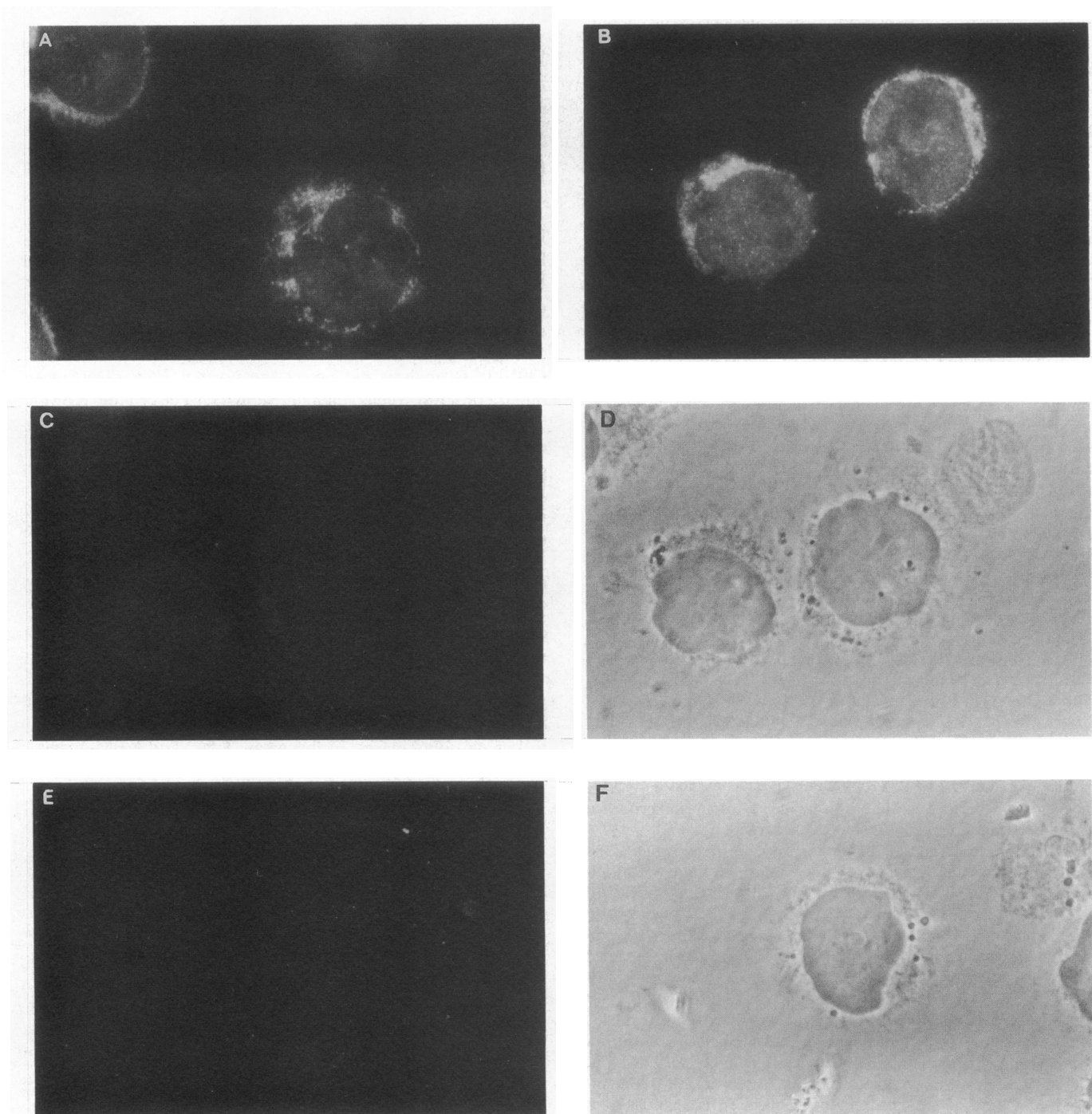


Figure 3. Indirect immunofluorescence of K562 cells. Primary antibodies were as follows: (A) anti-pEX4 antibody directed against the Abl COOH terminus, (B) rabbit polyclonal anti-Bcr antibody, (C) normal rabbit IgG, (D) phase contrast of C; (E) anti-pEX4 antibody preadsorbed with pEX4 fusion protein; and (F) phase contrast of E. $\times 1,000$.

confounding variable. Similarly, p190^{BCR-ABL} was found to be cytoplasmic in ALL-1 cells (Fig. 4 A). Negative controls with normal rabbit sera or with cognate peptide to block anti-pEX4 sera gave no significant fluorescence above background (Figs. 3 C and E, 4 C, and 5 C).

Murine c-Abl type IV protein exhibits predominantly nuclear localization in a hematopoietic cell line. In the mouse Ba/F3 cells that overexpress mouse c-Abl type IV protein (the myristoylated form of c-Abl) (22), c-Abl was found predomi-

nantly in the nucleus, (Fig. 6), but with some staining of the cytoplasm and with several cells exhibiting the prominent plasma membrane staining also seen with other myristoylated Abl proteins (22). Therefore, murine type IV c-ABL localization in the lymphoblastoid Ba/F3 cells was similar to that previously shown for this protein in fibroblasts (20).

The c-Abl protein exhibits predominantly cytoplasmic localization in human leukemic cell lines. The Ph¹-negative human myeloid leukemia cell lines (HL-60 and KG-1) demonstrated a

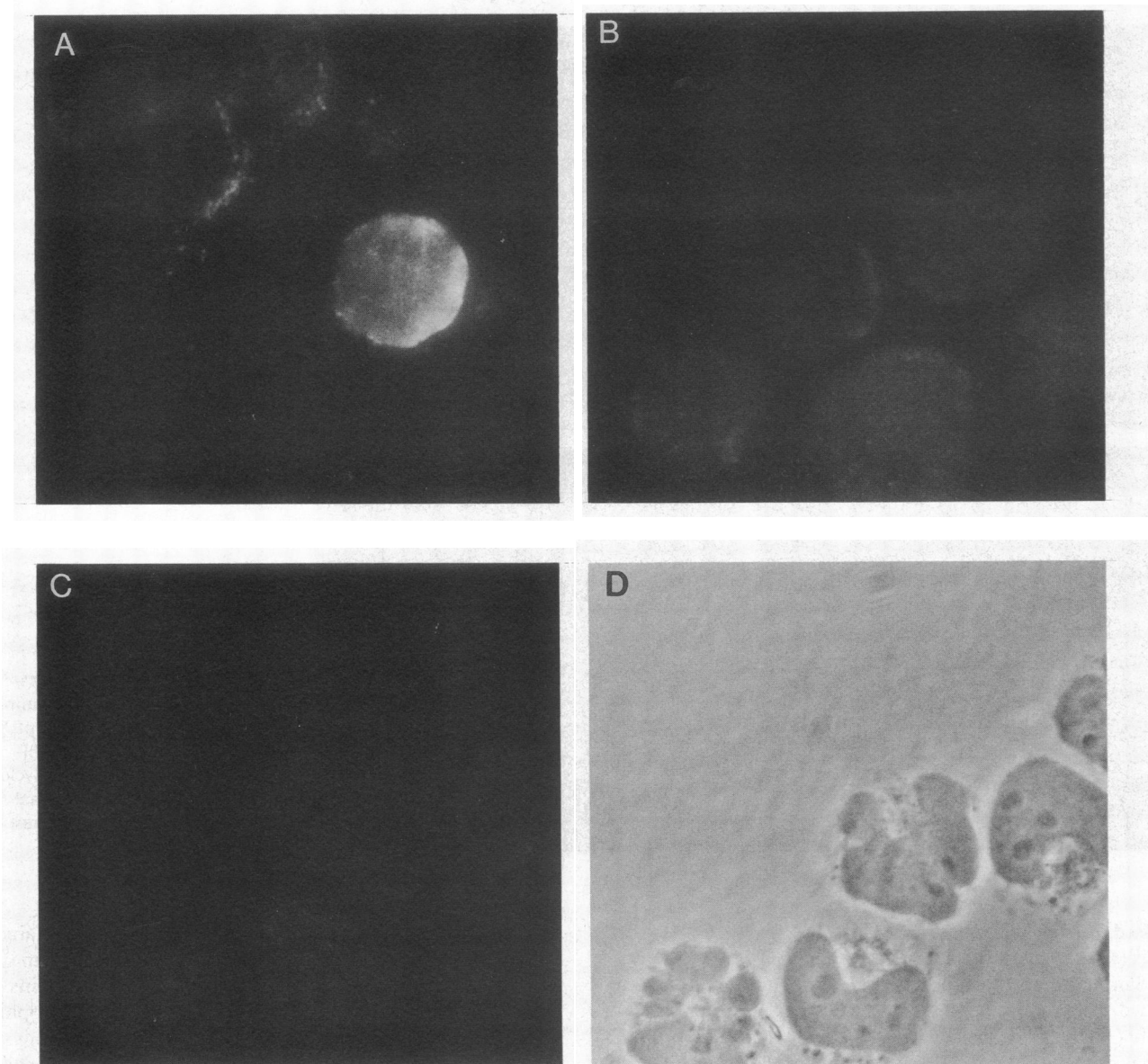


Figure 4. Indirect immunofluorescence of ALL-1 cells. Primary antibodies were as follows: (A) anti-pEX4 antibody directed against the Abl COOH terminus, (B) rabbit polyclonal anti-Bcr antibody, (C) normal rabbit IgG, and (D) phase contrast of C. $\times 1,000$.

signal for c-Abl which localized, however, mainly to the cytoplasm, with nuclear staining present at a lower level (HL-60, Fig. 7; KG-1 data not shown).

The Bcr protein exhibits cytoplasmic localization in leukemic cell lines. The immunofluorescence reactions with the rabbit polyclonal anti-Bcr antibody revealed positive cytoplasmic signals for Bcr protein in Ph¹-negative myeloid leukemic cell lines (HL-60 and KG-1) (Fig. 8). (In the Philadelphia-positive K562 and KBM-5 cell lines, the signals detected by this antisera were also cytoplasmic, but probably represent the p210^{BCR-ABL} protein which is expressed at high levels [Figs. 3 B and 5 B].) The p190^{BCR-ABL}-positive line (ALL-1) showed only a weakly positive cytoplasmic reaction (Fig. 4 B). In the latter cell line, the anti-Bcr sera would be expected to discern only the normal Bcr protein, and not p190^{BCR-ABL}, since both of our anti-Bcr antibodies were raised against a BCR region that is deleted from p190^{BCR-ABL} (33, 41). Control experiments with

normal rabbit IgG demonstrated no significant fluorescence (Figs. 3 C, 4 C, 5 C, and 8 B). Thus, the normal Bcr protein is cytoplasmic in the Ph¹-positive and Ph¹-negative cell lines tested.

Detection of c-Abl, Bcr, and Bcr-Abl proteins in fresh samples and correlation with myeloid differentiation. Using immunohistochemistry techniques and fresh Ph¹-positive CML cells, the anti-pEX4 Abl sera (Fig. 9, C-E), as well as the monoclonal anti-Bcr (7C6) reacted (Fig. 10, C and D), with decreasing intensity, as the cells progressed from myeloblasts and promyelocytes to myelocytes, metamyelocytes, and mature polymorphonuclear cells. Since CML cells express high levels of p210^{BCR-ABL}, and since both of these antisera detect this protein, these results suggest that the levels of p210^{BCR-ABL} are higher in earlier stages of myeloid maturation. The signal for Bcr-Abl was cytoplasmic in all of these cells, and there was no apparent change in subcellular localization as the disease pro-

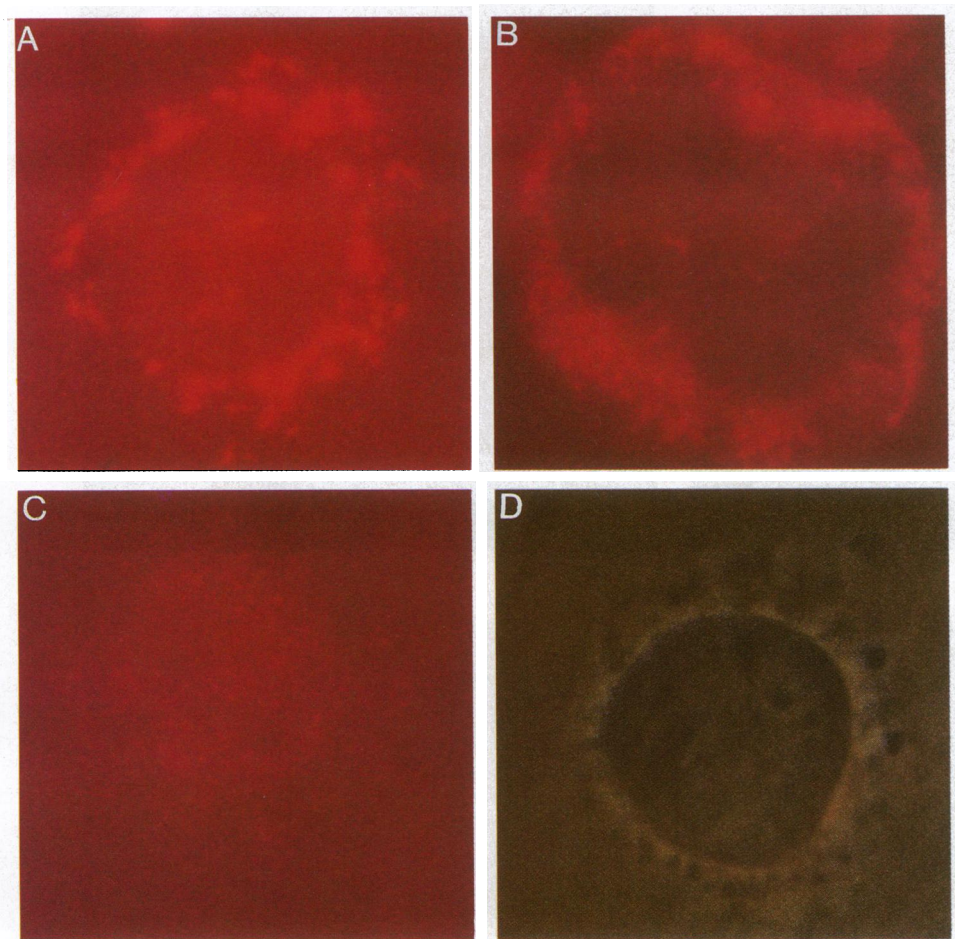


Figure 5. Indirect immunofluorescence of KBM-5 cells. Primary antibodies were as follows: (A) anti-pEX4 antibody directed against the Abl COOH terminus; (B) rabbit polyclonal anti-Bcr antibody, (C) normal rabbit IgG, and (D) phase contrast of C. $\times 1,000$.

gressed from chronic phase (Figs. 9, C and D and 10 C) to blast crisis (Figs. 9 E and 10 D).

Samples derived from bone marrow of normal donors reflected the same pattern as CML cells i.e., the expression of c-Abl (Fig. 9 A), as well as Bcr (Fig. 10 A) at an early maturational stage, as determined with the use of the anti-pEX4 sera and the monoclonal anti-Bcr (7C6), respectively. However, there was a weaker signal than seen in CML samples. Cells derived from patients with Ph¹-negative acute myelogenous leukemia (50–70% blasts), also revealed weakly positive staining for c-Abl and Bcr solely in cells in the early myeloblastic stages (Figs. 9 B and 10 B). No Abl or Bcr signal was detected in normal control peripheral blood samples which contain no immature myeloid cells (data not shown). The localization was predominantly cytoplasmic in all cells tested for both Bcr and c-Abl. In all experiments with fresh cells in which the anti-pEX4 sera was used, control experiments with cognate peptide for anti-pEX4 sera, as well as with normal rabbit sera demonstrated no significant signal. Similarly, in experiments in which the monoclonal anti-Bcr (7C6) was used, controls with isotypic antibody consistently demonstrated no significant reactions (data not shown).

To further confirm that a reduction in Bcr-Abl protein levels accompanied myeloid differentiation, immunoblotting was performed. Peripheral blood cells from a CML myeloid blast crisis patient were separated by Percoll gradient. The original sample contained 60% blasts, the fraction at 1.045 g/ml con-

tained 98% blasts, and the fraction at 1.080 g/ml contained 92% neutrophils. As can be seen in Fig. 11, blast cells (lane 2) had considerable higher levels of Bcr-Abl and Abl proteins as compared to unfractionated leukocytes (lane 1) or neutrophils (lane 3).

In vitro differentiation induction of HL-60 cells and correlation with the presence of Bcr and Abl proteins. Immunofluorescence techniques using anti-pEX4 sera and monoclonal anti-Bcr (7C6) sera, demonstrated that DMSO-induced granulocytic maturation of HL-60 cells was accompanied by a significant decrease in Abl and Bcr positivity, respectively (Fig. 12 A, B, D, and E). Control experiments with normal rabbit IgG, and isotypic antibody, respectively, showed no significant fluorescence (data not shown).

Discussion

Subcellular localization of p210^{BCR-ABL}, p190^{BCR-ABL}, and the normal Bcr and Abl proteins. Abl belongs to a family of tyrosine protein kinase enzymes and, in general, these types of proteins are membrane receptors or cytoplasmic, with the latter presumed to function as signal transducing molecules. In humans, two alternative forms of the normal c-Abl protein exist (type 1a and 1b); they differ in their amino terminal 26 and 44 amino acids (42, 43). These proteins are highly homologous to the murine c-Abl type I and IV proteins, respectively (44). Structural and sequence analyses have revealed several

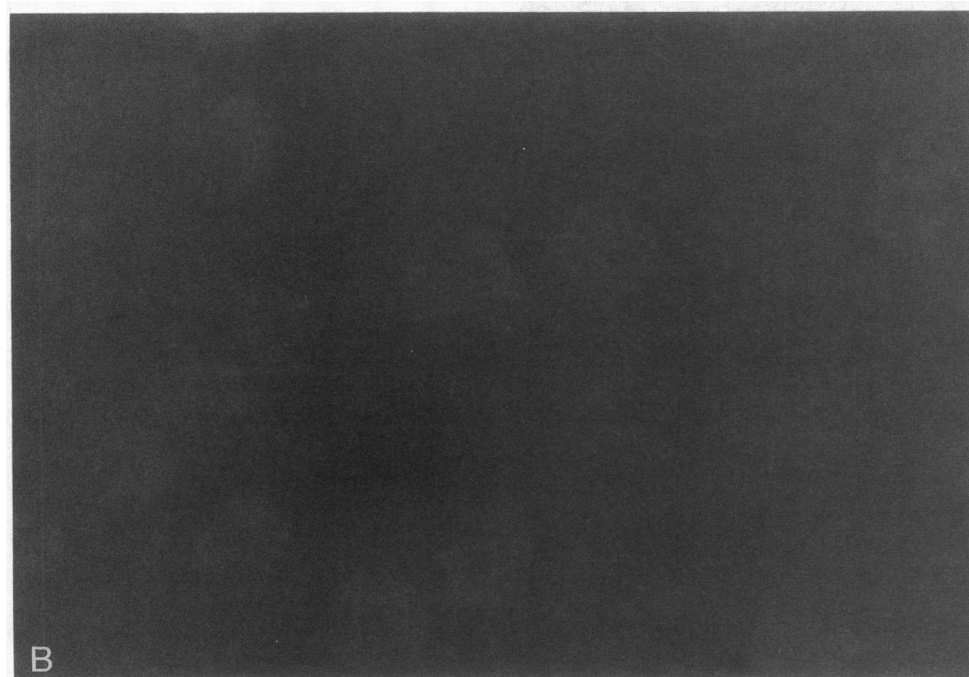
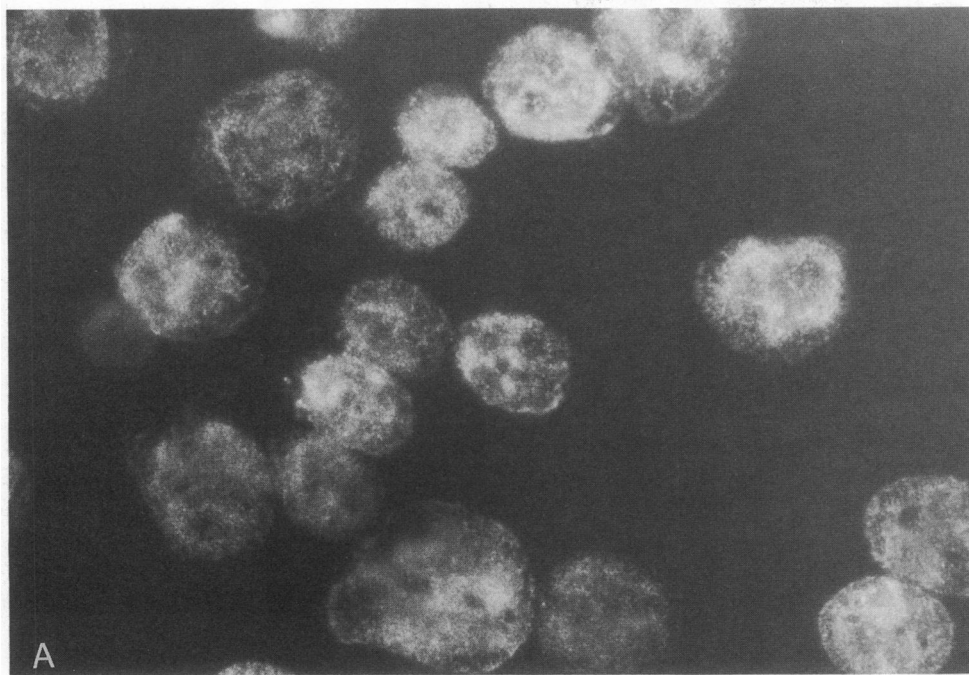


Figure 6. Indirect immunofluorescence of Ba/F3 cells. Primary antibodies were as follows: (A) anti-pEX4 antibody directed against the Abl COOH terminus and (B) normal rabbit IgG. $\times 1,000$.

NH₂- and COOH-terminal features that may provide clues to c-Abl subcellular localization and function. For instance, mouse c-Abl IV is myristoylated on the NH₂-terminus, whereas c-Abl I is predicted not to be myristoylated (45). Human c-Abl 1b also contains an NH₂-terminus glycine residue and a putative myristoylation sequence. Attachment of the myristoyl moiety is involved in targeting cellular proteins to associate with the plasma membrane as exemplified by Gag-Abl (viral Abl) proteins, which are NH₂-myristoylated and distribute at least in part to the cellular membrane (36, 46, 47). Other studies indicate that it is the carboxy-terminal of c-Abl that is critical in determining subcellular localization. This re-

gion contains domains that confer on the mammalian c-Abl protein the ability to bind both DNA (potential nuclear localization) (48) and cytoskeletal actin microfilaments (potential cytoplasmic localization) (20, 49). In addition, the carboxy terminus of *Drosophila* Abl dictates proper localization to axons in central nervous system cells (50).

Recently, Van Etten and colleagues (20) have demonstrated that when the murine type IV c-Abl protein is overexpressed in NIH3T3 fibroblasts, it is found to be largely nuclear, with smaller amounts in cytoplasm, plasma membrane, and associated with the actin cytoskeleton. A COOH-terminal motif that is unique to c-Abl and not found in Src or other tyrosine

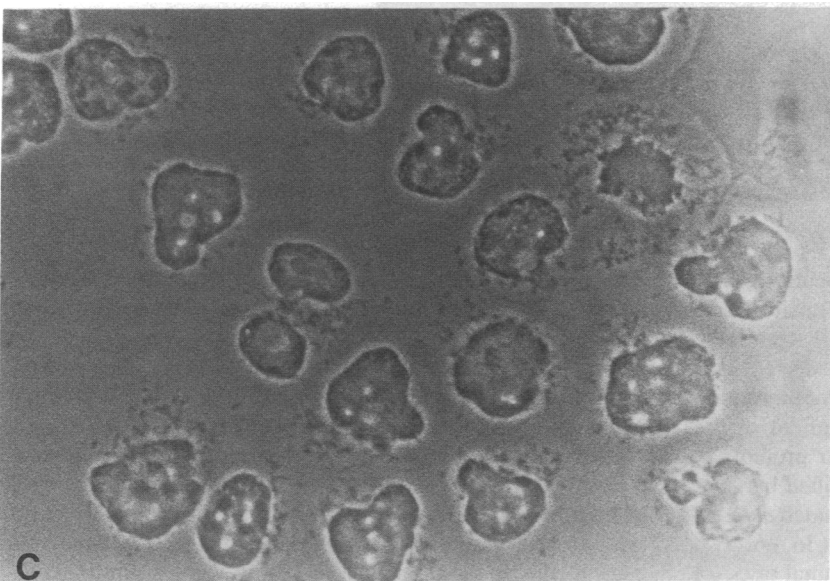
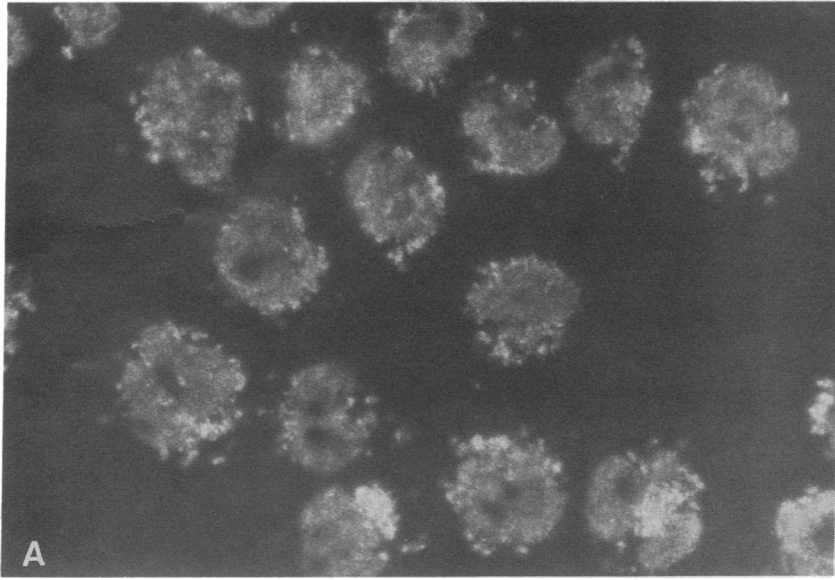


Figure 7. Indirect immunofluorescence of HL-60 cells. Primary antibodies were as follows: (A) anti-pEX4 antibody directed against the Abl COOH terminus, (B) normal rabbit IgG, and (C) phase contrast of B. $\times 1,000$.

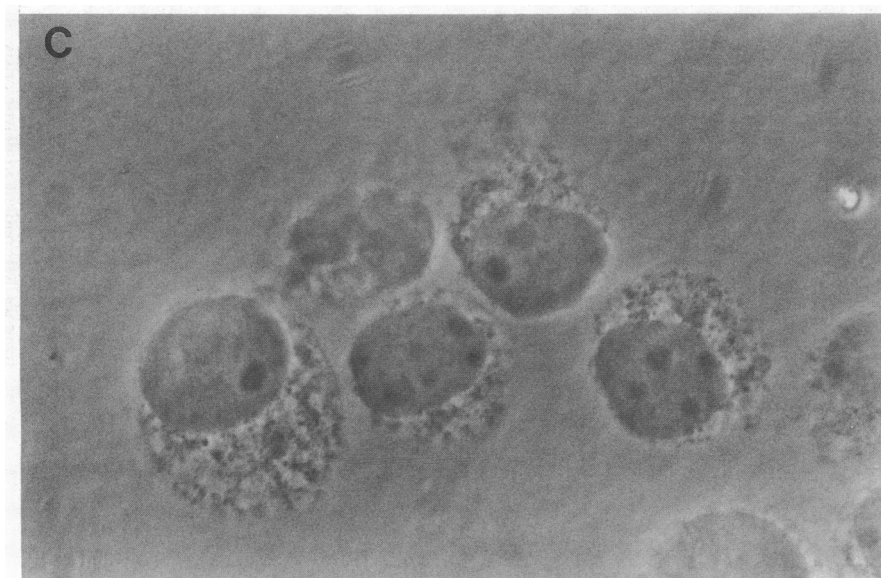
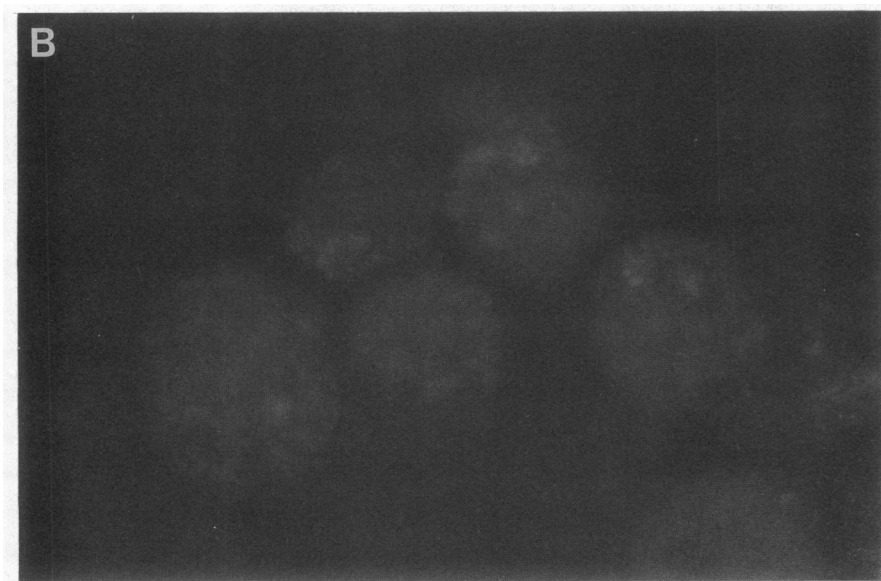
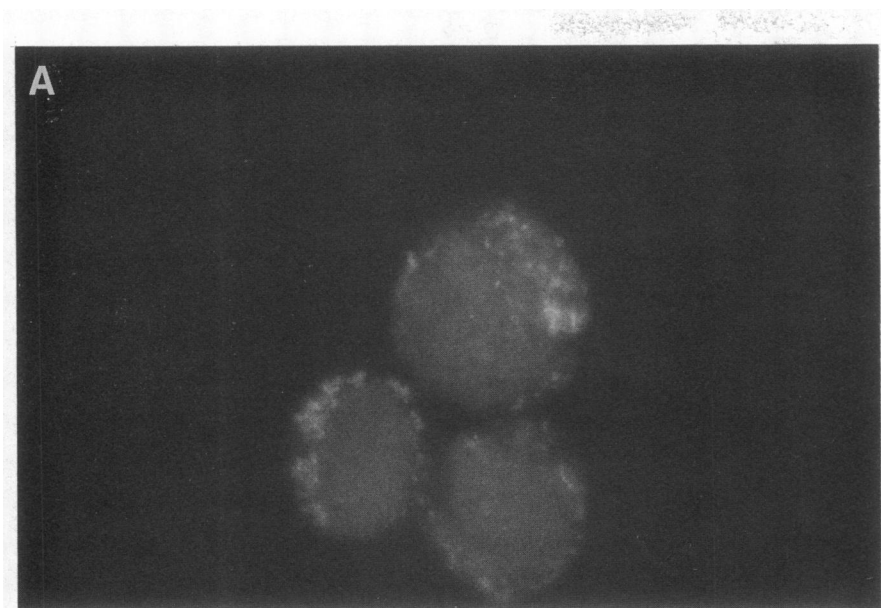


Figure 8. Indirect immunofluorescence of HL-60 cells. Primary antibodies were as follows: (A) rabbit polyclonal anti-Bcr antibody; (B) normal rabbit IgG, and (C) phase contrast of B. $\times 1,000$.

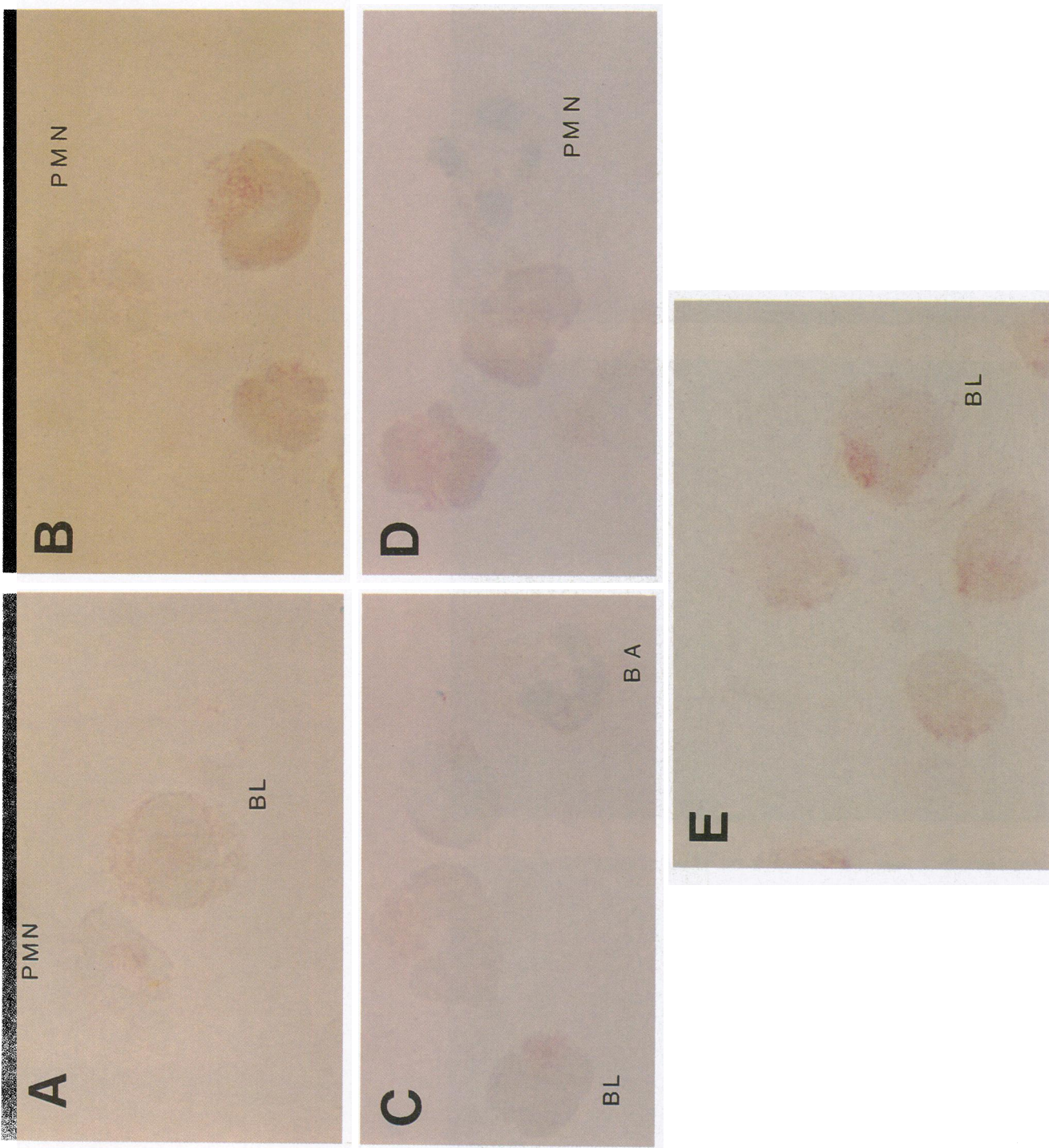


Figure 9. Immunohistochemical localization of c-Abl and Bcr-Abl proteins in fresh samples. Primary antibody was anti-pEX4 antibody directed against the Abl COOH terminus. (A) Normal bone marrow cells; the figure depicts a blast and a polymorphonuclear cell. (B) Cells derived from an AML patient (Ph¹ negative); the figure depicts an immature cell, a blast, and a polymorphonuclear cell. (C and D) Cells derived from two chronic-phase CML patients; C depicts two early immature cells and a polymorphonuclear cell, and D depicts a blast, two additional immature cells, and a band. (E) Cells derived from a CML patient in blastic crisis, all blasts.

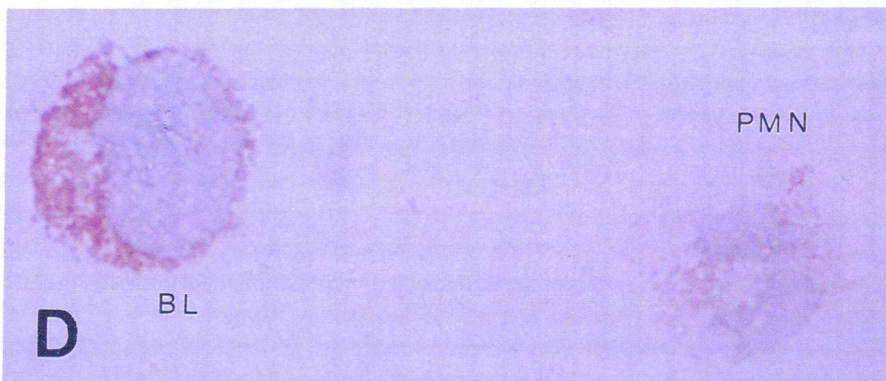
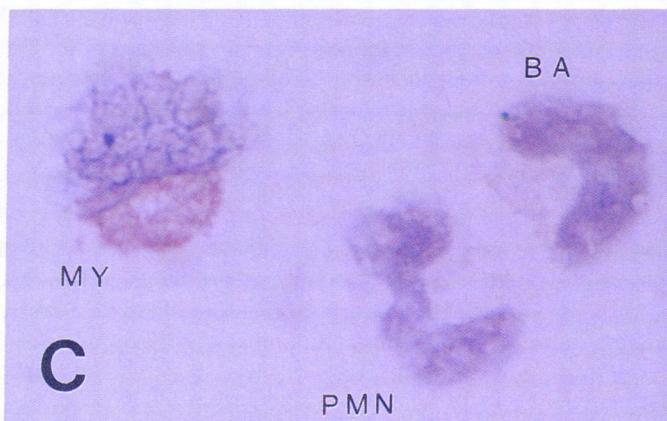
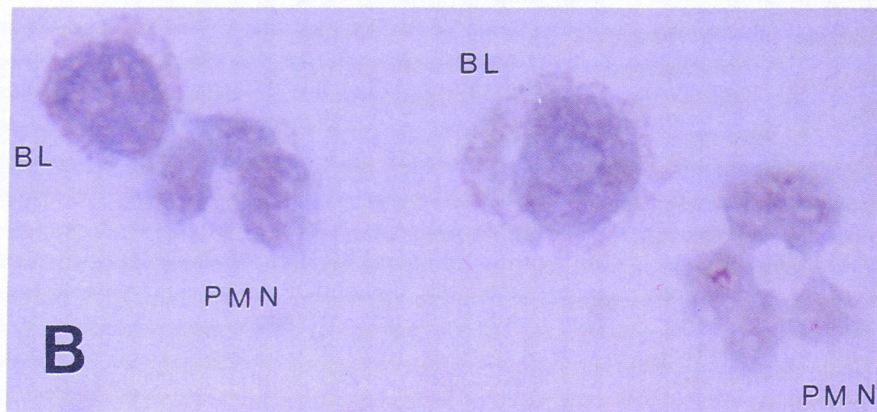
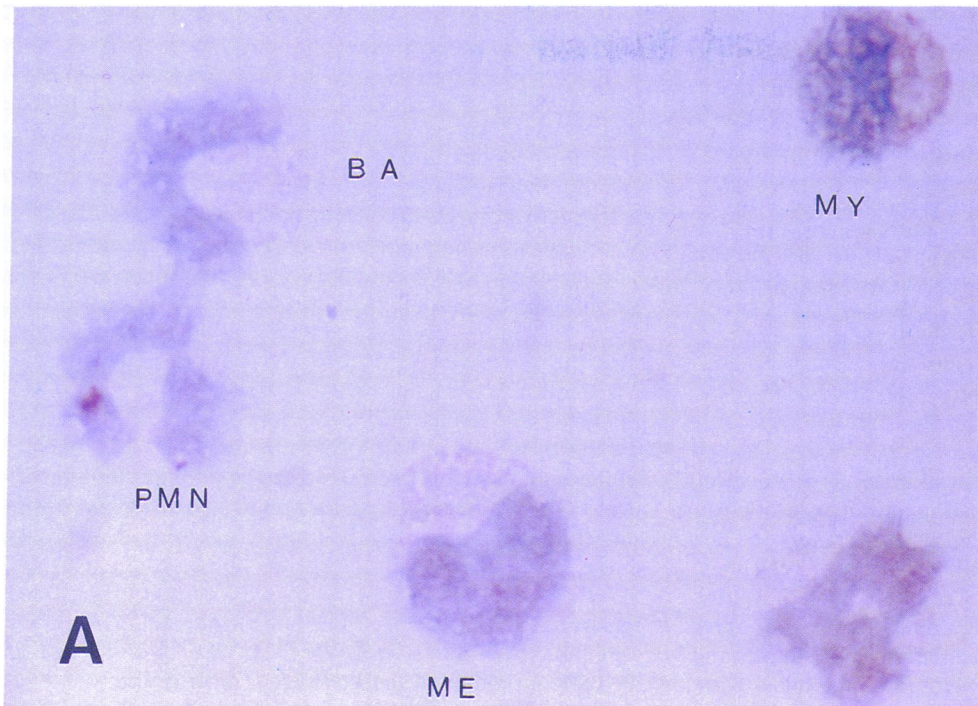


Figure 10. Immunohistochemical localization of Bcr and Bcr-Abl proteins in fresh samples. Primary antibody was monoclonal anti-Bcr (7C6) antibody. (A) Normal bone marrow cells; the figure depicts an immature cell, most probably a myelocyte, a metamyelocyte, a band, and a polymorphonuclear. (B) Cells derived from an AML patient (Ph¹ negative); the figure depicts two blasts and two polymorphonuclears. (C) Cells derived from a chronic phase CML patient; the figure depicts a myelocyte, a band, and a mature polymorphonuclear. (D) Cells derived from a CML patient in blastic crisis; the figure depicts a blast and a mature polymorphonuclear.

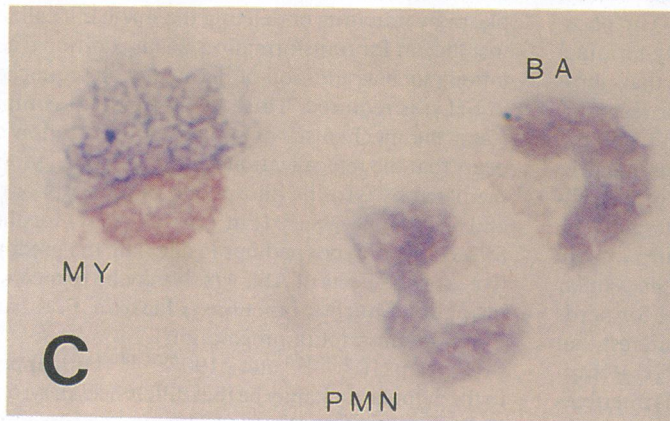
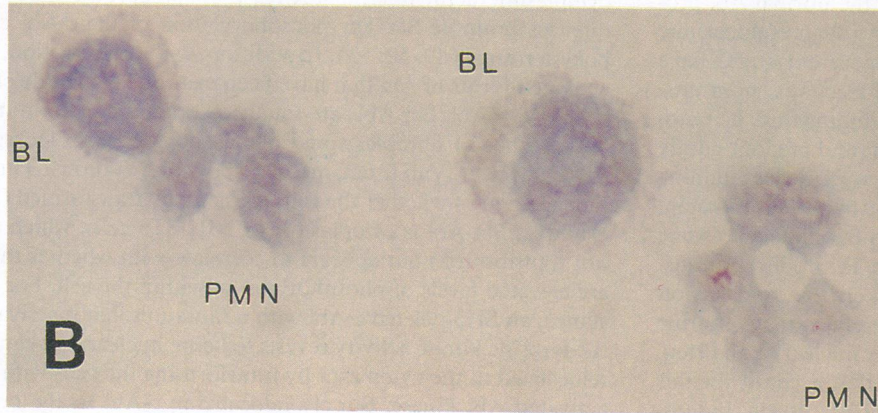
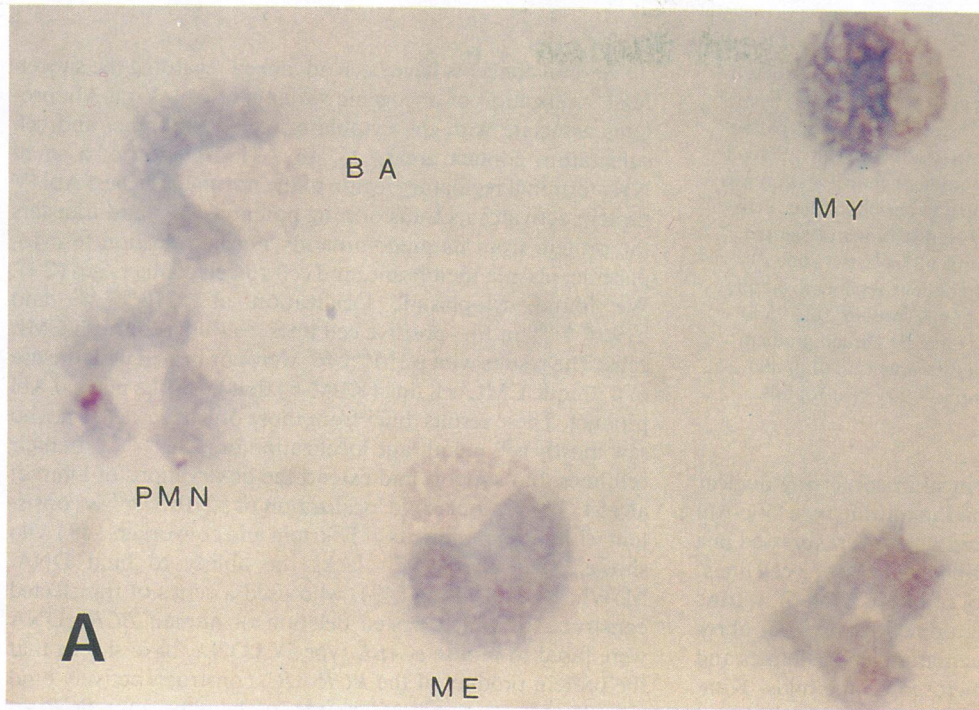


Figure 10. Immunohistochemical localization of Bcr and Bcr-Abl proteins in fresh samples. Primary antibody was monoclonal anti-Bcr (7C6) antibody. (A) Normal bone marrow cells; the figure depicts an immature cell, most probably a myelocyte, a metamyelocyte, a band, and a polymorphonuclear. (B) Cells derived from an AML patient (Ph¹ negative); the figure depicts two blasts and two polymorphonuclears. (C) Cells derived from a chronic phase CML patient; the figure depicts a myelocyte, a band, and a mature polymorphonuclear. (D) Cells derived from a CML patient in blastic crisis; the figure depicts a blast and a mature polymorphonuclear.

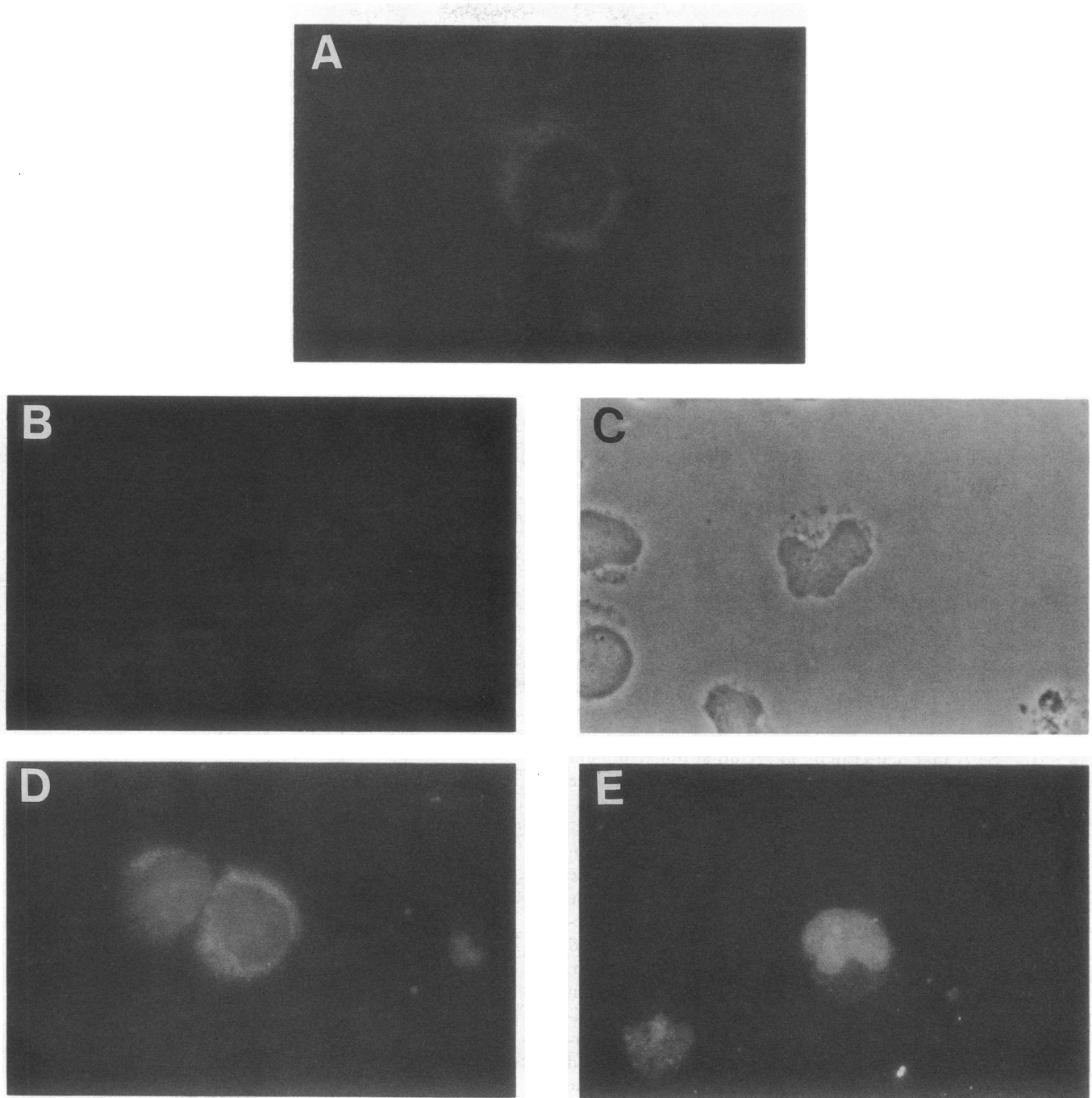


Figure 12. Indirect immunofluorescence of HL-60 cells induced by DMSO to undergo granulocytic differentiation. Primary antibodies were as follows: (A–C) anti-pEX4 antibody directed against the Abl COOH terminus; A depicts undifferentiated cells, B shows DMSO-exposed differentiated cells, and C depicts phase contrast of B. (D and E) Monoclonal anti-Bcr (7C6) antibody; D shows undifferentiated cells, and E depicts DMSO-exposed differentiated cells. $\times 1,000$. The secondary antibody against anti-Abl sera (A–C) was rhodamine conjugated; no background fluorescence is seen with this fluorochrome. The secondary antibody used with anti-Bcr sera was fluorescein conjugated (D and E). Background fluorescence of the nucleus is seen with the use of this fluorochrome and is of the same intensity in cells exposed to anti-Bcr sera or control isotypic antibody (data not shown).

come lineage committed and eventually differentiate into functional, morphologically distinct end-stage cells. This process is accompanied by the coordinate expression of numerous genes. For example, sustained expression of *c-MYB* blocks the differentiation of Friend murine erythroleukemia cells (58), whereas a reduction of *c-MYB* expression by specific antisense oligodeoxynucleotide blocks the proliferation of normal hu-

man bone marrow mononuclear cells in progenitor colony culture assay (59), suggesting that *c-MYB* plays a role in controlling proliferation and differentiation of hematopoietic cells. Similarly, K562 cells express high levels of *c-MYC* and *c-MYB* mRNA; differentiation along the erythroid lineage induced by 1- β -D-arabinofuranosylcytosine, daunomycin, or hemin is accompanied by a decrease in *c-MYC* and *c-MYB* transcript lev-

els (60, 61). A decrease in *c-MYC* and *c-MYB* also accompanies myeloid differentiation of HL-60 cells (62) and a murine myeloid leukemic cell line (63). Thus, it seems that the down-regulation of *c-MYC* and *c-MYB* mRNA may be a significant event in myeloid and erythroid maturation.

Little data exists regarding the role of Bcr and Abl proteins in hematopoiesis. Our observations indicate that the expression of both Bcr and Abl proteins among normal myeloid cells is inversely related to maturation; thus, myeloblasts and promyelocytes are highly positive for Bcr and Abl proteins, whereas polymorphonuclear cells are weakly positive. These findings have been substantiated by the *in vitro* differentiating experiments on HL-60 cells. Their maturation toward the granulocytic pathway was accompanied by a decrease in Bcr and Abl protein levels.

P210^{BCR-ABL} is also expressed at higher levels in immature myeloid CML cells than in more mature elements, but the signal appears stronger than that of normal Abl in normal bone marrow myeloid precursors, and does not disappear completely with full myeloid maturation. The mechanism by which both the Bcr-Abl, Bcr, and c-Abl proteins are down regulated upon differentiation of myeloid cells and cell lines is not known. This might occur at the transcriptional or posttranscriptional level. Bcr-Abl proteins are expressed off the normal Bcr promoter, so this might reflect the normal pattern of expression of this promoter. Interestingly, the same drop in P210 expression occurs upon differentiation to the metamyelocyte and neutrophil stage in CML mice, where P210 is expressed off a retroviral promoter, so this phenomenon might be of a more general nature (Van Etten, R. A., unpublished observation). Alternatively, previous experiments demonstrating a decrease in p210^{BCR-ABL} after hemin-induced erythroid differentiation of K562 cells (61, 64) suggest a translational mechanism since *BCR-ABL* mRNA remains unchanged. However, the relationship between erythroid differentiation of K562 cells and Bcr-Abl expression may be more complex, since the use of certain alternate differentiation agents such as cytosine arabinoside does not alter p210^{BCR-ABL} levels (61). In regard to c-Abl, some antisense experiments (65) implicate a lineage-specific function of c-Abl in myeloid development, so our findings would suggest that this action of Abl is quite early in differentiation. The presence of elevated levels of an aberrant Bcr-Abl protein (which is known to have a high constitutive tyrosine phosphokinase enzymatic activity) may, therefore, account for the discordant maturation (66) and resultant expansion of the myeloid compartment, which is the phenotypic hallmark of chronic phase CML.

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