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Alternative Forms of the *BCR-ABL* Oncogene Have Quantitatively Different Potencies for Stimulation of Immature Lymphoid Cells

JAMI McLAUGHLIN,[1] ERIN CHIANESE,[1,2] AND OWEN N. WITTE[1,2]*

Department of Microbiology and Molecular Biology Institute[1] and Howard Hughes Medical Institute,[2] University of California, Los Angeles, 405 Hilgard Avenue, Los Angeles, California 90024-1570

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The Philadelphia chromosome (t9:22;q34:q11) is found in more than 90% of patients with chronic myelogenous leukemia, in 10 to 20% of patients with acute lymphocytic leukemia, and in 1 to 2% of patients with acute myelogenous leukemia. Alternative chimeric oncogenes are formed by splicing different sets of *BCR* gene exons on chromosome 22 across the translocation breakpoint to a common set of *ABL* oncogene sequences on chromosome 9. This results in an 8.7-kilobase mRNA that encodes the P210 *BCR-ABL* gene product commonly found in patients with chronic myelogenous leukemia or a 7.0-kilobase mRNA that produces the P185 *BCR-ABL* gene product found in most Philadeliphia chromosome-positive patients with acute lymphocytic leukemia. To compare the efficiency of growth stimulation by these two proteins, we derived cDNA clones for each with identical 5′ and 3′ untranslated regions and expressed them from retrovirus vectors. Matched stocks were compared for potency to transform immature B-lymphoid lineage precursors. The growth-stimulating effects of P185 for this cell type were found to be significantly greater than those of P210. Structural changes in *BCR* may regulate the effectiveness of the *ABL* tyrosine kinase function, as monitored by lymphocyte growth response. Changes in mitogenic potency may help to explain the more acute leukemic presentation usually associated with expression of the P185 *BCR-ABL* oncogene.

The family of human leukemias associated with the specific balanced translocation known as the Philadelphia chromosome (Ph1;t9:22;q34:q11) (37, 43, 45) provides a useful system to investigate the relationship between structural changes in an oncogene and the effects of these changes on cell transformation specificity and efficiency (3, 7). A large body of data has accumulated to support the idea that the Ph1 translocation of chronic myelogenous leukemia (CML) occurs in pluripotent stem cells (3, 16, 33). Cells of multiple hematopoietic lineages can be shown to contain the Ph1 during the chronic phase of the disease. Although the progeny of the affected stem cells in CML are clearly abnormal in subtle aspects of growth kinetics and growth factor requirements (13, 17, 51), they are capable of differentiation. This situation is quite different from that seen in the blood as the disease advances to a blast crisis stage, in which a single myeloid or lymphoid progenitor cell type grows aggressively.

The mechanisms that relate this translocation to the activation of a specific oncogene have been established for CML. Almost all of the breakpoints on chromosome 22 fall within a 5-kilobase-pair (kbp) segment known as *bcr* (breakpoint cluster region; 18). This region is surrounded by the coding exons of a gene of unknown function, also called *BCR* (19, 20). *BCR* is one member of a family of four closely related genes distributed along chromosome 22 (9). The breakpoints found on chromosome 9 are more widely distributed, but all fall upstream of a specific exon of the *ABL* oncogene, known as the common acceptor exon or exon 2 (1, 2). Normal expression of the *ABL* oncogene uses alternative first-exon choices joined to this common acceptor exon to generate a family of mRNAs (1, 50). In CML, this results in a predicted large transcription product initiating on chromosome 22 and crossing over the translocation breakpoint to the *ABL* sequences. Splicing reduces this structure to a final mRNA of about 8.7 kb which encodes the P210 *BCR-ABL* gene product (10, 25, 35, 50). The similarities in structure and activated tyrosine kinase activity of this altered form of the *ABL* oncogene to the *gag-abl* fusion product expressed by the Abelson murine leukemia virus (11, 23, 25) suggested that the *BCR-ABL* gene fusion would be a functional oncogene. Recent studies using long-term marrow culture systems and retrovirus-mediated gene transfer have documented that P210 *BCR-ABL* can stimulate the growth of immature hematopoietic precursor cell types (34, 58).

A similar mechanism is used in a distinctive clinical entity known as Ph1-positive acute lymphocytic leukemia (ALL). This very aggressive leukemia can occur in children or adults and initially presents with a high leukocyte count and a monomorphic blood picture, without evidence of cell differentiation from the leukemic clone (40). It is not clear whether the lymphoid cell type that predominates in the peripheral blood was the original cell in which the translocation occurred or whether the rapidly growing leukemic cells were derived from a more primitive cell in which the Ph1 arose (52). It is noteworthy that ALL patients induced to remission lose the Ph1 from the peripheral blood, whereas chronic-stage CML patients in remission from chemotherapy treatment are usually positive for the Ph1 in the peripheral blood (40).

Cells from Ph1-positive ALL patients frequently contain a serologically cross-reactive, activated ABL tyrosine kinase with an estimated molecular weight of 185,000 (4, 6, 27). In some series (5, 14, 44, 48), up to 50% of clinically diagnosed Ph1-positive ALL patients have the structural changes in *bcr* to generate the P210 gene product, whereas in other series almost all Ph1-positive ALL patients either express the P185 gene product or show rearrangement in the first *BCR* intron (C. T. Denny, N. Shah, S. Ogden, C. William, T. McConnell, W. Crist, A. Carroll, and O. N. Witte, submitted for publication; S. S. Clark, V. Najfeld, L. Dow, W. Crist, R.

* Corresponding author.

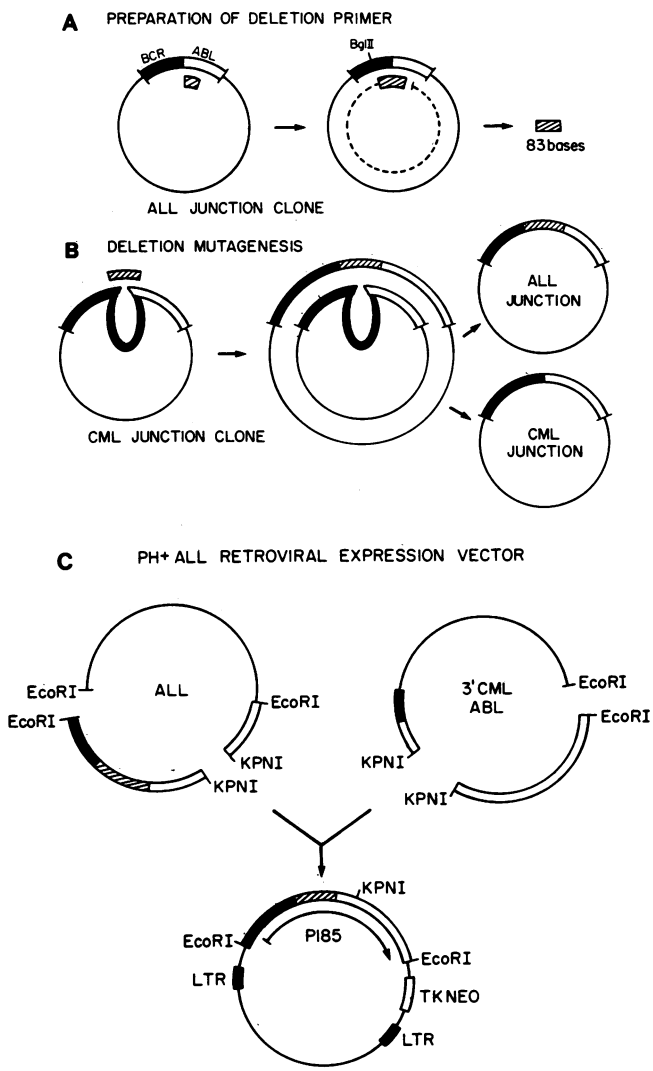

FIG. 1. Construction of P185- and P210-expressing retrovirus vectors. To create P210- and P185-expressing retrovirus vectors with identical 5′ and 3′ untranslated regions, a precise internal deletion of *BCR* sequences from a P210 cDNA corresponding to the additional splicing difference that creates P185 was carried out. (A) A deletion primer was prepared by extending a 20-mer ABL-negative strand-specific oligonucleotide of sequence 5′-TGATGCT CAAAGTCAGATGC-3′ after hybridization to an M13 sense strand of clone A27, representing 491 bp from the ALL-specific *BCR-ABL* junction (7). The duplex DNA was cut with *Bgl*II, and the released 83-bp single-stranded fragment was isolated on a denaturing polyacrylamide gel. (B) The 83-bp fragment was used to prime an M13 sense strand of clone 161 (35), representing 93 bp of 5′ untranslated region and 4.5 kbp of the P210-coding region, which includes all of the *BCR* segment and about two-thirds of the *ABL* segment. The resulting phages, containing either the ALL-specific deletion or the parental-strand CML-specific *BCR-ABL* junction, were screened by differential hybridization with a BCR oligonucleotide from the deleted region (35) and confirmed by nucleotide sequence analysis, using the dideoxy-chain termination method (46). (C) The ALL- or CML-specific junctions represented in the *Eco*RI-to-*Kpn*I fragment were constructed in the *Eco*RI site of retrovirus vector pMV6tkneo (32) by a three-part ligation, using the *Kpn*I-to-*Eco*RI fragment of clone 215, which contains most of the ABL-coding sequence and about 500 bp of 3′ untranslated region (35). Only the final construction encoding the P185 gene product is shown.

Champlin, and O. N. Witte, Mol. Diagn. Hum. Cancer, in press; V. Najfeld, J. Cuttner, A. Figur, E. Kawasaki, O. N. Witte, and S. S. Clark, submitted for publication). The reason for this discrepancy is not clear. This P185 product results from the same type of mechanism as that used in CML except that the position of the breakpoint on chromosome 22 falls upstream of the *bcr* region in the first intron of the *BCR* gene (7, 15, 21, 53). This results in a smaller fusion protein with an amino-terminal segment representing the first coding exon of *BCR* and the set of coding exons derived from the *ABL* gene starting at the common acceptor exon (2). In several recent reports (5, 28; Najfeld et al., submitted), patients with Ph1-positive acute myelogenous leukemia (a rare leukemia representing only 1 to 2% of all patients with the disease) were shown to express the specific mRNA and 185,000-molecular-weight tyrosine kinase form of the *BCR-ABL* gene found in Ph1-positive ALL. Since either of these acute leukemias could be confused with CML that had progressed to the blast crisis stage with a myeloid or lymphoid predominance, the association of the more acute diseases with the smaller BCR-ABL protein and message should be useful diagnostically.

These different BCR-ABL protein structures may change the effectiveness of the ABL tyrosine kinase as a growth stimulus. To test this idea, we prepared retrovirus constructs to express the P210 and P185 gene products in mouse bone marrow cultures under selective conditions that favor the growth of immature lymphoid cell precursors (34, 56). Although both gene products could stimulate a similar cell type to become clonally dominant in culture, P185 was a more potent growth stimulus than P210.

MATERIALS AND METHODS

Cell culture and virus stocks. Retrovirus constructions using the vector pMV6tkneo (32), expressing the P210 *BCR-ABL* or P185 *BCR-ABL* gene product (see legend to Fig. 1 for procedures), were cotransfected with Moloney murine leukemia virus DNA into NIH 3T3 cells and selected for expression of the linked bacterial neomycin gene; clonal isolates were preliminarily screened for production of released viral RNA by slot blot analysis (34). Selected clones were further analyzed by effectiveness of transfer of ABL-specific kinase activity as described in the legend to Fig. 2.

Culture conditions selective for the long-term propagation of immature elements of the B-cell lineage derived from murine bone marrow and the conditions for virus infection were as described previously (34, 54–56, 58) and in the figure legends. All experiments were initiated with bone marrow from 4-week-old BALB/c mice.

The granulocyte-macrophage colony-stimulating factor (GM-CSF)-dependent cell line DA3.15 was obtained from Donna Rennick and cultured as previously described (39) in 100 U of murine GS-CSF per ml in RPMI 1640 medium with 5% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.).

Nucleic acid and protein analyses. Analyses of *BCR-ABL* retroviral genome structure, mRNA expression, and production of immunoprecipitable tyrosine autophosphorylation activity were all performed by standard procedures (26, 34, 47, 57). Specific antisera, probes, and conditions are given in the figure legends.

RESULTS

P185-expressing retrovirus stimulates the same cell type as does P210 in a long-term lymphoid bone marrow culture

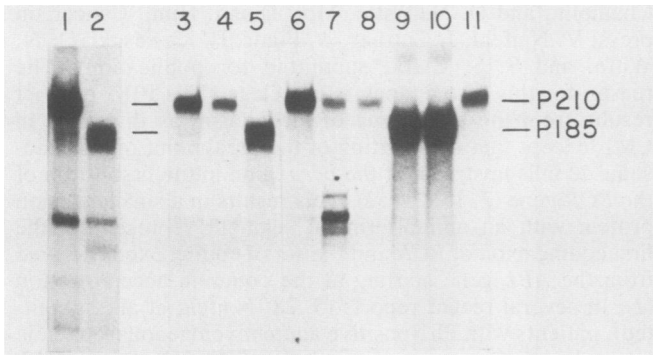

FIG. 2. Determination of efficiency of retrovirus infection. Independently harvested virus stocks of P210 (lanes 3, 4, 6, 7, 8, and 11) or P185 (lanes 5, 9, and 10) rescued with Moloney murine leukemia virus were evaluated for efficiency of virus transfer and expression by infection of subconfluent monolayers (about 10^6 cells in a 10-cm-diameter dish) of NIH 3T3 cells with 1.5 ml of each stock in the presence of 8 μg of polybrene per ml. Cells were harvested at 72 h postinfection and extracted for immunoprecipitation autophosphorylation assay, using 10 μCi of [γ-^{32}P]ATP and 10 mM Mn^{2+} (26, 57) with rabbit anti-pEX-5 antibody, which is reactive with sequences in the carboxy-terminal region of both proteins (6, 24). Lanes 1 and 2, P185 and P210, respectively, isolated from clonal NIH 3T3 lines harboring one copy of either genome (about 2×10^6 cells each) and autophosphorylated as described above to serve as size and relative concentration standards. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as previously described (26). Exposure time was 4 h.

system. Previous work had demonstrated that expression of the P210 protein from a retrovirus vector under the control of a Moloney leukemia virus-derived long-terminal-repeat segment could stimulate growth of immature elements of the B-cell lineage when the cells were plated under long-term culture conditions (34). This effect was evidenced by the outgrowth, 4 to 5 weeks after infection of mouse bone marrow, of clonal cell lines that had the phenotypic characteristics of progenitor or pre-B cells. These lines all showed J_H rearrangements, germ line kappa alleles, high levels of terminal deoxynucleotidyl transferase expression, B-lineage-specific markers like the B220 protein, and active transcription of immunoglobulin μ-related messages. They grew poorly in agar, and most could not induce tumors in syngeneic intraperitoneal challenges (34; see below and Table 1). Our overall impression was that P210 was a relatively weak growth stimulus for this cell type and required secondary events to complement progression of the clonal lines to a full malignant phenotype, analogous to the clinical progression of chronic-phase CML to blast crisis.

To accurately compare the quantitative potency of P185 with that of P210 in this system, we needed to prepare retrovirus constructions that differed only in the *BCR* coding sequences. Because recent data from our laboratory demonstrated a dramatic effect of the 5′ untranslated region of *BCR* on translational efficiency (A. J. Muller, J. McLaughlin, and O. N. Witte, manuscript in preparation), we planned our constructions to produce expression of P185 or P210 from cDNA inserts with identical 5′ and 3′ untranslated segments (Fig. 1). This was accomplished by site-specific mutagenesis of a P210-expressing cDNA clone to precisely delete the segment of *BCR* not present in P185, as defined from previous cloning and sequencing analyses (7, 15).

Virus stocks were prepared by transfection of each construct into NIH 3T3 cells with Moloney murine leukemia virus full-length DNA to produce packaging proteins and polymerase gene functions. Since neither Moloney murine leukemia virus nor *BCR-ABL* oncogenes transform NIH 3T3 cells (10), we selected for G418 resistance dependent on the *cis* expression of a neomycin gene expressed from an internal thymidine kinase promoter in each *BCR-ABL* construct (Fig. 1; 34). Individual colonies were picked, expanded, and tested for release of viral RNA into virion particles in the supernatant by a rapid slot blot procedure (34). Variability in virus titer was observed, which was probably a reflection of the chronic toxic effect that members of the *ABL* oncogene family can have on fibroblast cell lines (10, 59). This finding necessitated screening many dozens of clones to select high-titer producers which could then be compared more quantitatively.

Selected stocks were tested for functional transfer of the *BCR-ABL* oncogene to fresh NIH 3T3 cell populations by virus infection, followed by extraction 72 h later for immunoprecipitation tyrosine autophosphorylation assay (26; Fig. 2). We found this to be the most accurate and reproducible way to compare functional virus titers. Selected stocks were diluted to equivalent levels of kinase transfer before use in the hematopoietic growth assays. Full-length P210 and P185 were accurately expressed from the retrovirus vectors. The P185 protein appeared as a close doublet of bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as has been described for the natural product expressed in material from Ph1-positive ALL patients and cell lines (6). The precise cause of this doublet of bands has not been identified.

When P185 stocks rescued with Moloney murine leukemia virus (P185/M) were used to infect mouse bone marrow in liquid culture, rapidly growing populations were obtained within 2 to 3 weeks in most cases. All of the cultures expressed high levels of P185 autokinase activity (Fig. 3A) and were clonal or pauciclonal, as monitored by viral integrations (Fig. 3B). Analysis of DNA rearrangements of the immunoglobulin heavy-chain loci and RNA expression showed that all cultures were predominantly of the immature pre-B-cell phenotype (Fig. 3B). All showed rearranged J_H regions on both alleles and many submolar bands, which indicated continuing rearrangement after virus infection and progression to clonality. All clones expressed the early B-lineage markers of immunoglobulin μ sterile transcript and B220 (Ly-B5), and four of six expressed mRNA for terminal deoxynucleotidyl transferase (Fig. 3C). This phenotype is indistinguishable from that previously reported for a series of transformants derived with P210/M stocks (34). The only difference observed was that the P185-infected cultures seemed to grow more rapidly, since abundant cell growth was seen in most cases by 3 weeks, compared with the 4 to 5 weeks previously observed with P210-infected cultures.

P185 is more effective than P210 as a growth stimulus for immature lymphoid cells. To quantitatively compare the potency of the *BCR-ABL* gene products, virus stocks that were carefully matched for equal effectiveness at transferring functional kinase activity (Fig. 2) were selected. Four independent experiments were conducted to evaluate the time course and frequency of transformation by each stock of target cells in 5×10^6 murine bone marrow cells plated in individual long-term cultures (Fig. 4). For example, P210/M stock (Fig. 2, lane 6) was matched against P185/M stock (lane 5) or against a 1:1.5 dilution of P185/M stock (lane 9). Most hematopoietic cells in the inoculum died during the first week to 10 days of culture, during which time a mixed-cell stromal layer developed that produced factors

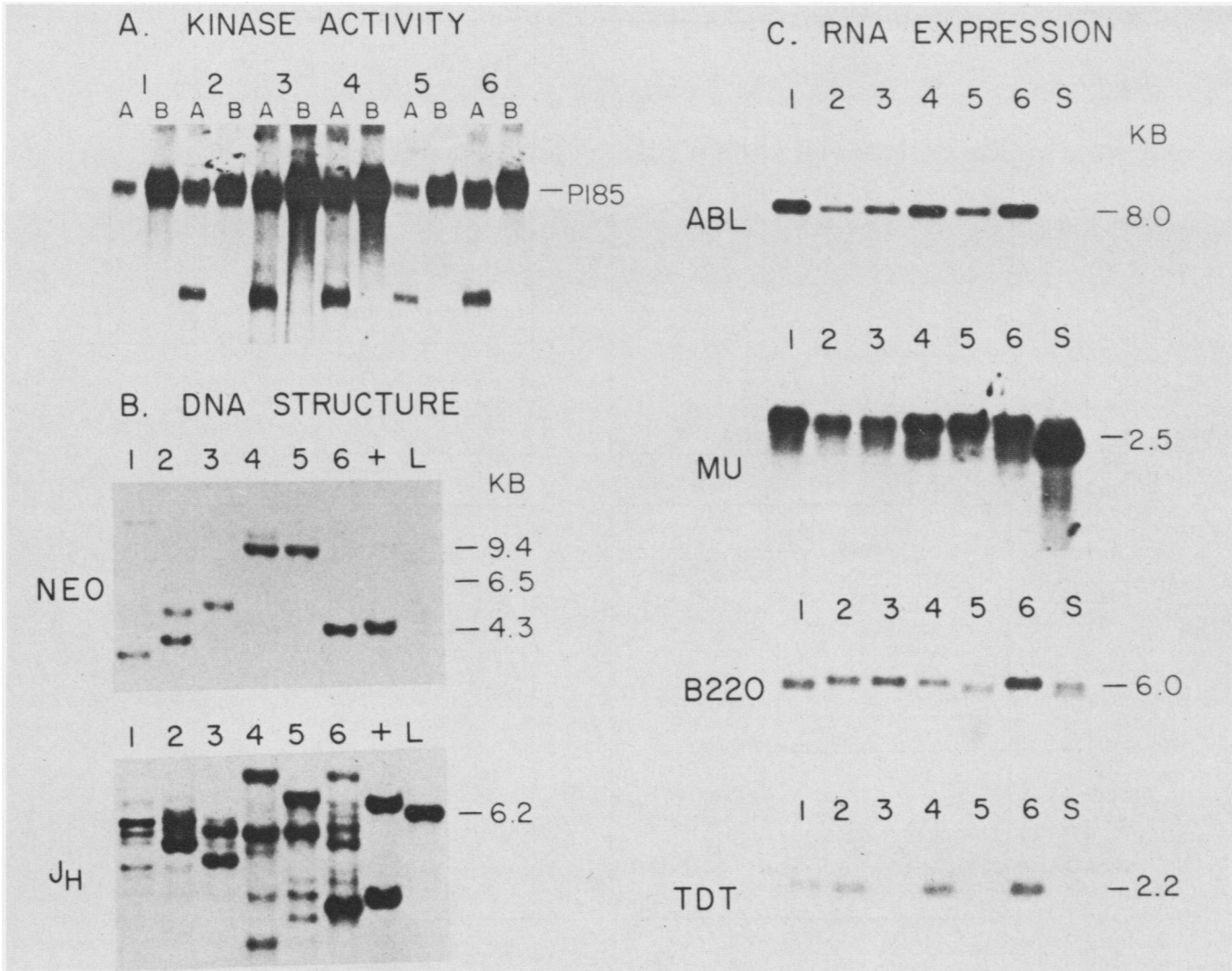

FIG. 3. Immature lymphoid phenotype of P185 transformants. (A) About 2×10^6 cells each of six P185 lymphoid transformants were extracted, immunoprecipitated for tyrosine-specific autophosphorylation assay, using rabbit anti-BCR (7) or rabbit anti-pEX-5 reactive with carboxy-terminal sequences of the ABL protein segment (24) (lanes B), and analyzed as described in the legend to Fig. 1. (B) High-molecular-weight DNAs isolated from the six P185 lymphoid transformants were digested with *Eco*RI and analyzed by DNA blot hybridization (lanes 1 through 6) with a 2-kbp fragment of the *tk-neo* insert (32) or a 1.8-kbp fragment of the murine J_H region (12); each was labeled by nick translation (41) to $>10^8$ cpm/μg. Positions of size markers from standard bacteriophage lambda DNA digests (data not shown) and of an unrearranged germ line J_H fragment of 6.2 kbp are noted. Other lanes: +, previously studied P210 *BCR-ABL* transformant with an integrated *neo* gene and rearranged J_H loci; L, control liver DNA negative for *neo* DNA or J_H rearrangements. (C) Expression of specific mRNAs monitored by blot hybridization on total RNA fractionated on a 1% agarose-formaldehyde gel (47). Probes included a 4.5-kbp *ABL* fragment to detect retrovirus-encoded *BCR-ABL* genomic RNA or mRNA (35), a 400-bp immunoglobulin μ constant-region probe (42), a fragment of the Ly-B5 (B220) cDNA (49), and a 2-kbp fragment of the cDNA encoding murine terminal deoxynucleotidyl transferase (TDT) (29). Each probe was labeled by nick translation to $>10^8$ cpm/μg. Approximate size designations are relative to published values; positions of the large and small rRNA subunits were visualized by ethidium bromide staining.

required for efficient outgrowth of lymphoid elements (22, 36, 56). Lymphoid precursor cells or their virus-infected counterparts then grew up above the background level of dying cells. The data in Fig. 4 are plotted to show the relative growth rates from less than or equal to 10^6 to 10^7 or more cells per 5-cm-diameter culture dish, starting on days 14 to 16, at which time significant numbers of lymphoid cells could be counted. Uninfected cultures and cultures infected with Moloney murine leukemia virus alone, set up identically, attained maximal densities of 1×10^6 to 2×10^6 cells per culture by 30 to 40 days. Cells from cultures transformed by P185 or P210 stocks showed larger average lymphoid cell sizes than did cells from uninfected cultures when high cell density was obtained, as was previously observed (34, 55).

Two clear differences are apparent from the combined data in Fig. 4. First, a much higher percentage of cultures infected with P185 reached high density (24 of 28, or 86%) than was found for P210-infected cultures (13 of 27, or 48%). Second, on average, the P185-infected cultures reached high cell density earlier (before 3 weeks), although a few P210-infected cultures reached 10^7 cells by days 17 to 21.

Because each of the experiments described above was dependent on the outgrowth of a dominant clone above the background of uninfected cells or other cells expressing P210

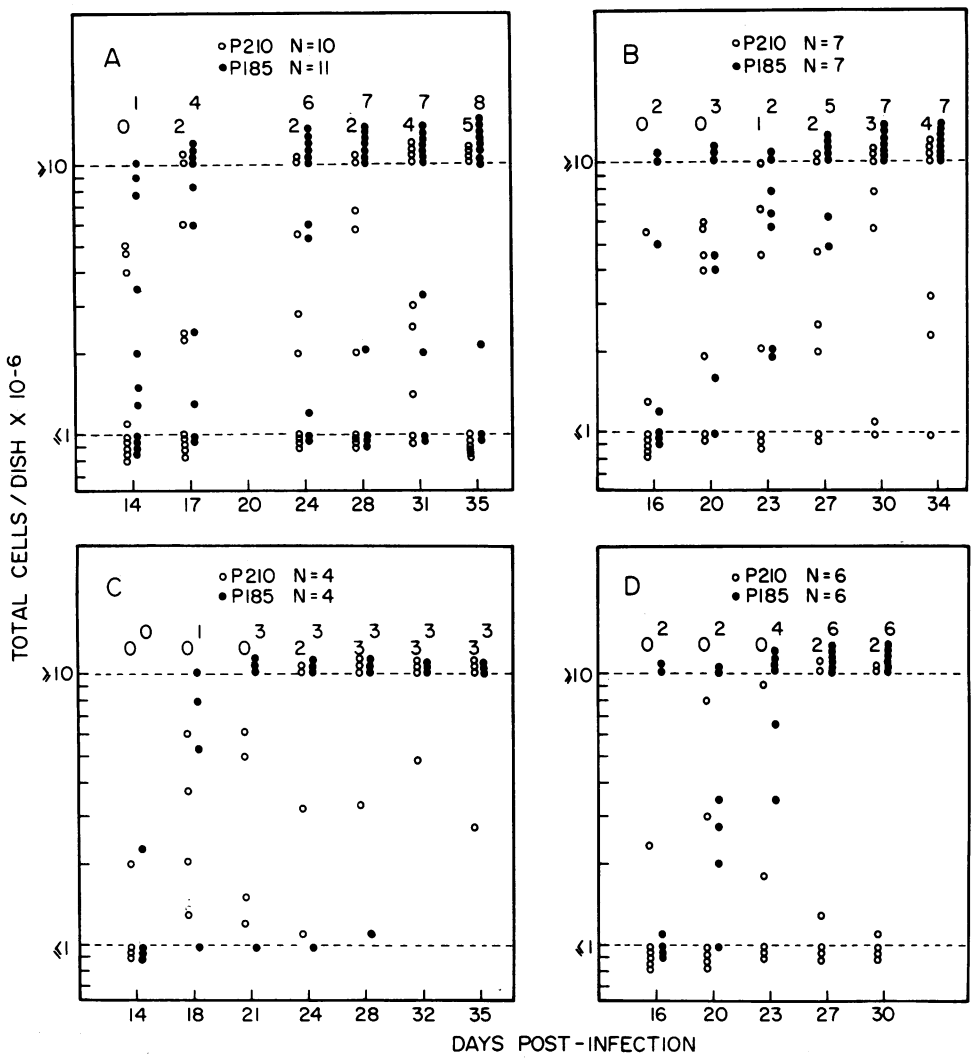

FIG. 4. Time course of lymphoid transformation. Four experiments (A to D) were conducted. P210/Mo and P185/Mo stocks, selected and diluted for equal efficieney of transfer of tyrosine kinase activity as described in the legend to Fig. 2, were compared. A total of 5×10^6 freshly explanted femoral bone marrow nucleated cells from 3- to 4-week-old BALB/c mice were infected with 2.5 ml of stock in the presence of 2.5 ml of complete medium with 10% fetal calf serum and polybrene (final concentration, 8 μg/ml) for 3 h at 37°C. Cells were washed and plated at 10^6/ml in 60-mm-diameter dishes under conditions previously established for the long-term culture of murine B-lymphoid elements (54, 56). Cultures were fed twice weekly, cell populations were agitated and sampled, and viable lymphoid cells were counted beginning on day 13 or 14 postinfection, at which time a stromal layer had formed and nonadherent cells were visable. Counts are total number of lymphoid cells per culture. The number of cultures infected with P210/Mo or P185/Mo is indicated at the top of each panel. Counts below 10^6 or above 10^7 are grouped. The number of cultures reaching $\geq 10^7$ is noted above each time point.

or P185, we were concerned that the rate of spread of *BCR-ABL* genomes in the mass culture system would be a significant factor. Since the retrovirus stocks were selected and matched for ability to acutely transfer P210 and P185, the possibility existed that differences in the Moloney helper virus titers could bias the results by changing the number of cells affected during secondary rounds of infection. Examination of the levels of reverse transcriptase in the matched sets of stocks showed that helper activity could vary by as much as three- to fivefold, but there was no consistently higher level for the P185 or P210 stocks among the sets we examined (data not shown).

To control for this possibility, we conducted a series of lymphoid transformation experiments in which we blended equal amounts of prematched stocks of P185 and P210 before infection of bone marrow and plating in long-term culture conditions. Whatever the differences in helper virus titers, under these conditions both P185 and P210 could spread with the same efficiency. A series of independent cultures as evaluated by autokinase assay for dominant expression of P210 or P185 or expression of a mixture of both proteins when each culture reached more than 10^7 total lymphoid cells. The results were striking in that 17 of 24 total cultures examined showed only P185, two showed P210, and five showed a mixture (Fig. 5 and data not shown). Evaluation of the integrated retroviral genomes in each population showed the expected diagnostic fragment length corresponding to a single P185 or P210 genome or a mixture of both, in agreement with the kinase results (data not shown).

As a final test to evaluate the blended stocks, we carried out a similar set of infections on the myeloid cell line DA3.15, which can be maintained in either interleukin-3 or

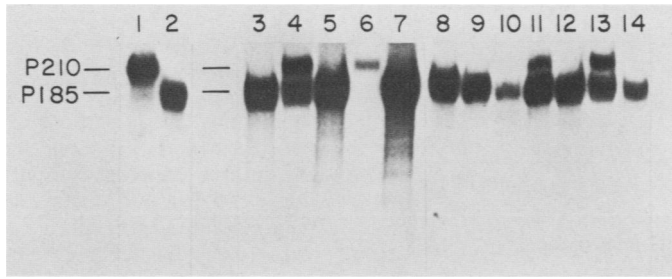

FIG. 5. Competiton between P185 and P210 during mixed infections of bone marrow lymphoid progenitors. Pretitered stocks of P185/Mo and P210/Mo selected for equivalent efficiencies of kinase transfer were mixed 1:1 and used to infect fresh bone marrow as described in the legend to Fig. 4. Cultures were fed, monitored for regrowth of lymphoid cells, and sacrificed when the total lymphoid population reached 10^7 or more cells per culture. Cells were extracted for tyrosine autophosphorylation assay as described in the legends to Fig. 2 and 3. Individual cultures (lanes 3 to 14) are shown, along with size standards of P185 (lane 2) and P210 (lane 1) from previously established clonal lines.

GM-CSF (39). Previous work in our laboratory (A. J. Muller, E. Chianese, and O. N. Witte, unpublished observations) had shown that P210 expression could render this cell line independent of GM-CSF, in a manner analogous to the effect of Abelson murine leukemia virus expression on certain interleukin-3-dependent lines (8, 38). DA3.15 cells were infected with the same P185-P210 mixed stocks used in the lymphoid transformation experiments, replated in growth factor-containing medium for 48 h to allow viral integration, expression, and spread, and then washed and replated in growth factor-free medium. Cultures were fed and harvested when individual plates contained more than 10^7 cells, generally by 2 to 3 weeks postinfection. Autokinase activity and DNA blot analysis to evaluate the dominant genome type showed that the vast majority of cultures (11 of 13) expressed both P185 and P210 (Fig. 6 and data not shown). Some cultures (Fig. 6, lane 2) showed higher levels of P185 kinase activity. This phenomenon appeared to be attributable to multiple independent clones growing in the same dish in some cases, as monitored by DNA blot analysis evaluating numbers of viral integrations (data not shown), and a preferential degradation of P210 during extraction and immunoprecipitation procedures when lysates of myeloid cell origin are used (J. Young and O. N. Witte, unpublished observations). Some clones (Fig. 6, lanes 3 and 4) represented doubly infected clonal lines, as evidenced by further single-cell subcloning and DNA blot analysis (data not shown). These results supported the idea that the P210 and P185 stocks were closely matched, since they were equally effective in transferring the viral genome in this culture model. Acquisition of growth factor independence could be generated with similar efficiency by both gene products, while the kinetics of lymphoid transformation were faster for the P185 gene product.

Expression of P185 is not sufficient for development of a fully transformed phenotype. P185 was found to be more efficient than P210 at expanding immature lymphoid precursors to clonal dominance in culture (Fig. 4 and 5). Previous analyses of P210-derived lymphoid lines had shown that these lines varied widely in ability to grow in agar and induce progressive lymphomas after intraperitoneal inoculation into syngeneic mice (34). P210 was perhaps necessary, but

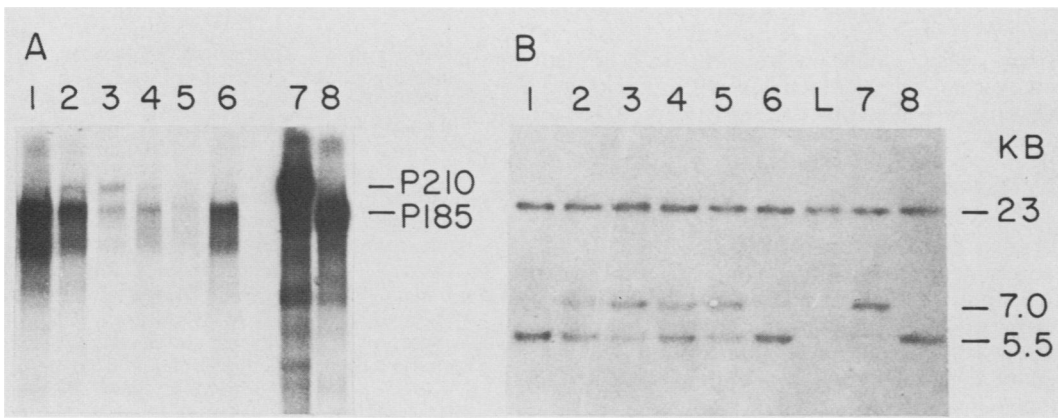

FIG. 6. Competition between P185 and P210 during infection of the GM-CSF-dependent cell line DA3.15. For each culture, 2×10^6 DA3.15 cells (39) grown in the presence of 100 U of murine GM-CSF per ml were infected with the same blended P185/Mo-plus-P210/Mo stocks used in the experiments shown in Fig. 5. Cells were plated at 10^6/ml for each culture for 48 h in the continued presence of GM-CSF and then washed and replated in medium without added growth factor. Cultures were fed twice weekly and observed for regrowth of factor-independent populations. Cells were harvested when the total culture reached $\geq 10^7$ cells and analyzed by immunoprecipitation tyrosine autophosphorylation assay for the presence of P185 and P210 (A) and DNA blot analysis (B) to evaluate the relative numbers of genomes of each virus. (A) Approximately 5×10^6 cells were extracted, processed, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as for Fig. 5. Individual cultures (lanes 1 to 6) are compared with clonal cell lines harboring either P210 (lane 7) or P185 (lane 8). (B) High-molecular-weight DNAs harvested from the same cultures were digested with *Eco*RI to release the 7.0-kbp P210 or 5.5-kbp P185 inserts from the integrated retrovirus structures. A 0.7-kbp *ABL Pst*I fragment labeled by nick translation to 10^8 cpm/μg was used as a probe. These samples were compared with clonal lines containing a single P210 (lane 7) or P185 genome (lane 8) and normal mouse liver DNA (lane L).

TABLE 1. Transformation phenotypes of P185 and P210 clonal lymphoid lines

Line	BCR-ABL expression	Growth in agar[a]	No. of mice positive after in vivo tumor challenge[b]	Latency (days)
102C2	P210	0	0	
97D6	P210	0	2	60–70
102C6	P185	0	0	
97D1	P185	19	2	35–65
97D5	P185	22	1	40
106-3	P185	20	2	55–60
106-5	P185	14	3	13–15
M4[c]	P210	33		

[a] Each line was plated in duplicate at 10^3 and 10^4 cells per 5-cm-diameter dish without feeder layers as previously described (34). Data are average percentages of cells developing into macroscopic colonies over a 14-day period.

[b] Cells (2×10^6) of each clonal line were injected intraperitoneally into three 4- to 6-week-old BALB/c mice bred in our colony. Animals were observed daily for signs of progressive lymphoma and evaluated by autopsy for enlarged spleen, lymph nodes, ascites, or tumor mass at the site of injection. Animals were monitored for at least 10 weeks before sacrifice if no gross pathology was evident.

[c] Described by McLaughlin et al. (34) and used as a positive control for the agar assay.

clearly not sufficient, for expression of a fully transformed phenotype.

A series of independently derived P210 or P185 clonal lymphoid lines was tested at 8 weeks after culture establishment for ability to grow in agar and tumorigenic potential (Table 1). All of the clonal lines expressed high levels of either P185 or P210 tyrosine kinase (Fig. 3 and 5). Although several of the P185-derived lines showed modest agar growth potential, only one line (106-5) rapidly induced syngeneic tumors. Several of the other lines generated tumors but with longer latency periods. P185 expression did not obligately correlate with agar colony-forming ability or tumor progression. Although P185 was more effective than P210 in stimulating growth of lymphoid precursors in vitro and somewhat more efficient at in vivo tumor challenge (Table 1), expression of P185 alone was not sufficient for a fully transformed phenotype in every case. Additional genetic events are probably necessary to complement either P185 or P210 for tumor progression.

DISCUSSION

The results presented here support the concept that P185 provides a more potent growth stimulus for immature lymphoid cells than does P210. This increased potency does not appear to represent a gross difference between P210 and P185 in levels of stable protein or cell localization. Neither protein is myristylated, and both have half-lives of about 4 h in lymphoid cell lines (J. McLaughlin, S. Clark, A. M. Pendergast, and O. N. Witte, unpublished observations). One interpretation of these combined data would be that the P185 kinase is more effective at phosphorylation of a critical substrate required for unregulated growth that is found in all cell types affected by the *BCR-ABL* gene family.

The increased mitogenic potency of P185 in this lymphoid culture system may relate to a relative tissue-type specificity. Although both genomes seem roughly equivalent in ability to eliminate the requirement for GM-CSF for growth of DA3.15 cells, we have not successfully achieved efficient transformation of myeloid progenitor cells in long-term culture systems. Using the same long-terminal-repeat-driven P210 *BCR-ABL* virus used in this study and infection of established myeloid cultures, we surprisingly recovered very primitive lymphoid precursor cells that displayed resistance to corticosteroids present in the culture medium (58). It is not clear whether the long-terminal-repeat-driven constructs are poorly expressed in myeloid progenitors or whether these constructs are toxic and selected against during the lengthy culture procedures. Further analysis of the potential variation in tissue specificity for P185 and P210 will await new vector constructions, using alternative promoters to increase the range of expression and vary the quantitative level of mRNA produced.

Additional studies on the pathogenesis of the Ph1-positive leukemias will concentrate on the interaction of the *BCR-ABL* gene products with other oncogene alterations during the process of tumor progression. A variety of secondary mutations, including activations of *ras* family oncogenes (30), could be involved. We have recently developed an in vitro rodent fibroblast model which demonstrates a synergistic action of $P210^{BCR\text{-}ABL}$ with the *myc* oncogene (31a). Interestingly, *myc* oncogene expression is high in lymphoid blast crisis CML, whereas expression of P53, another nuclear oncogene, is high in some myeloid CML blast crisis cases (31). Even if the P185 gene product serves as a better mitogenic signal for some cell types, it is unlikely to be the only genetic change responsible for full expression of the malignant phenotype in acute leukemia associated with the Ph1 chromosome.

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LITERATURE CITED

1. **Ben-Neriah, Y., A. Bernards, M. Paskind, G. Q. Daley, and D. Baltimore.** 1986. Alternative 5'-exons in c-*abl* mRNA. Cell **44**:577–586.
2. **Bernards, A., C. M. Rubin, C. A. Westbrook, M. Paskind, and D. Baltimore.** 1987. The first intron in the human c-*abl* gene is at least 200 kilobases long and is a target for translocations in chronic myelogenous leukemia. Mol. Cell. Biol. **7**:3231–3236.
3. **Champlin, R., and D. Golde.** 1985. Chronic myelogenous leukemia: recent advances. Blood **65**:1039–1047.
4. **Chan, L. C., K. K. Karhi, S. I. Rayter, N. Heisterkamp, S. Eridani, R. Powles, S. D. Lawler, J. Groffen, J. G. Foulkes, M. F. Greaves, and L. M. Wiedemann.** 1987. A novel *abl* protein expressed in Philadelphia chromosome positive acute lymphoblastic leukemia. Nature (London) **325**:635–637.
5. **Chen, S. J., G. Flandrin, M.-T. Daniel, F. Valeusi, L. Baranger, D. Grausz, A. Berenheim, Z. Chen, F. Sigaux, and R. Berger.** 1988. Philadelphia-positive acute leukemia: lineage promiscuity and inconsistently rearranged breakpoint cluster region. Leukemia **2**:261–273.
6. **Clark, S. S., J. McLaughlin, W. N. Crist, R. Champlin, and O. N. Witte.** 1987. Unique forms of the *abl* tyrosine kinase distinguish Ph^1-positive CML from Ph^1 positive ALL. Science **235**:85–88.
7. **Clark, S. S., J. McLaughlin, M. Timmons, A. M. Pendergast, Y. Ben-Neriah, L. W. Dow, W. Crist, G. Rovera, S. D. Smith, and O. N. Witte.** 1988. Expression of a distinctive *BCR-ABL* oncogene in Ph1-positive acute lymphocytic leukemia (ALL). Science **239**:775–777.

8. **Cook, W. D., D. Metcalf, N. A. Nicola, A. W. Burgess, and F. Walker.** 1985. Malignant transformation of a growth factor-dependent myeloid cell line by Abelson virus without evidence of an autocrine mechanism. Cell **41**:677–683.
9. **Croce, C. M., K. Huebner, M. Isobe, E. Fainstain, B. Lifshitz, E. Stivelman, and E. Canaani.** 1987. Mapping of four distinct BCR-related loci chromosome region 22q11: order of BCR loci relative to chronic myelogenous leukemia and acute lymphoblastic leukemia breakpoints. Proc. Natl. Acad. Sci. USA **84**:7174–7178.
10. **Daley, G. Q., J. McLaughlin, O. N. Witte, and D. Baltimore.** 1987. The CML-specific P210 *bcr/abl* protein, unlike v-*abl*, does not transform NIH-3T3 fibroblasts. Science **237**:532–537.
11. **Davis, R. L., J. B. Konopka, and O. N. Witte.** 1985. Activation of the c-*abl* oncogene by viral transduction or chromosomal translation generates altered c-*abl* proteins with similar in vitro kinase properties. Mol. Cell. Biol. **5**:204–213.
12. **Early, P., H. Huang, M. Davis, K. Calame, and L. Hood.** 1980. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: V_H, D and J_H. Cell **19**: 981–992.
13. **Eaves, A. C., J. D. Cashman, L. A. Gaboury, D. K. Kalousek, and C. J. Eaves.** 1986. Unregulated proliferation of primitive chronic myeloid leukemia progenitors in the presence of normal marrow-adherent cells. Proc. Natl. Acad. Sci. USA **83**:5306–5310.
14. **Erickson, J., C. A. Griffin, A. ar-Rushdi, M. Valtieri, J. Hoxie, J. Finan, B. Emanuel, G. Rovera, P. C. Nowell, and C. M. Croce.** 1986. Heterogeneity of chromosome 22 breakpoint in Philadelphia-positive (Ph+) acute lymphoblastic leukemia. Proc. Natl. Acad. Sci. USA **83**:1807–1811.
15. **Fainstein, E., C. Marcelle, A. Rosner, E. Canaani, R. P. Gale, O. Dreazen, S. D. Smith, and C. M. Croce.** 1987. A new fused transcript in Philadelphia chromosome positive acute lymphocytic leukemia. Nature (London) **330**:386–388.
16. **Fialkow, P., P. Martin, V. Najfeld, G. Penfold, R. Jacobson, and J. Hansen.** 1981. Evidence for a multistep pathogenesis of chronic myelogenous leukemia. Blood. **58**:158–163.
17. **Gordon, M. Y., C. R. Dowding, G. P. Riley, J. M. Goldman, and M. F. Greaves.** 1987. Altered adhesive interactions with marrow stroma of hematopoietic progenitor cells in chronic myeloid leukemia. Nature (London) **328**:342–344.
18. **Groffen, J., J. R. Stephenson, N. Heisterkamp, A. de Klein, C. R. Bartram, and G. Grosveld.** 1984. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell **36**:93–99.
19. **Hariharan, I. K., and J. M. Adams.** 1987. cDNA sequence for human *bcr*, the gene that translocates to the *abl* oncogene in chronic myeloid leukaemia. EMBO J. **6**:115–119.
20. **Heisterkamp, N., K. Stam, J. Groffen, A. de Klein, and G. Grosveld.** 1985. Structural organization of the *bcr* gene and its role in the Ph^1 translocation. Nature (London) **315**:758–761.
21. **Hermans, A., N. Heisterkamp, M. von Lindern, S. van Baal, D. Meijer, D. van der Plas, L. M. Wiedemann, J. Groffen, D. Bootsma, and G. Grosveld.** 1987. Unique fusion of *bcr* and c-*abl* genes in Philadelphia chromosome positive acute lymphoblastic leukemia. Cell **51**:33–40.
22. **Hunt, P., D. Robertson, D. Weiss, D. Rennick, F. Lee, and O. N. Witte.** 1987. A single bone-marrow-derived stromal cell type supports the *in vitro* growth of early lymphoid and myeloid cells. Cell **48**:997–1007.
23. **Kloetzer, W., R. Kurzrock, L. Smith, M. Talpaz, M. Spiller, J. Gutterman, and R. Arlinghaus.** 1985. The human cellular *abl* gene product in the chronic myelogenous leukemia cell line has an associated tyrosin protein kinase activity. Virology **140**: 230–238.
24. **Konopka, J., R. Davis, S. Watanabe, F. Ponticelli, L. Schiff-Maker, and O. N. Witte.** 1984. Only site-directed antibodies reactive with the highly conserved *src* homologous region of the v-*abl* protein neutralize kinase activity. J. Virol. **51**:223–232.
25. **Konopka, J. B., S. M. Watanabe, and O. N. Witte.** 1984. An alteration of the human c-*abl* protein in K562 leukemia cells unmasks associated tyrosine kinase activity. Cell **37**:1035–1042.
26. **Konopka, J. B., and O. N. Witte.** 1985. Detection of c-*abl* tyrosine kinase activity in vitro permits direct comparison of normal and altered *abl* gene products. Mol. Cell. Biol. **5**: 3116–3123.
27. **Kurzrock, R., M. Shtalrid, P. Romero, W. S. Kloetzer, M. Talpas, J. M. Trujillo, M. Blick, M. Beran, and J. V. Gutterman.** 1987. A novel c-*abl* protein product in a Philadelphia-positive acute lymphoblastic leukemia. Nature (London) **325**:631-635.
28. **Kurzrock, R., M. Shtalrid, M. Talpaz, W. S. Koetzer, and J. V. Gutterman.** 1987. Expression of c-*abl* in Philadelphia-positive acute myelogenous leukemia. Blood **70**:1584–1588.
29. **Landau, N. R., T. P. St. John, I. L. Weissman, S. C. Wolf, A. E. Silverstone, and D. Baltimore.** 1984. Cloning of terminal transferase cDNA by antibody screening. Proc. Natl. Acad. Sci. USA **81**:5836–5840.
30. **Liu, E., B. Hjelle, and J. M. Bishop.** 1988. Transforming genes in chronic myelogenous leukemia. Proc. Natl. Acad. Sci. USA **85**:1952–1956.
31. **Lubbert, M., C. W. Miller, L. Crawford, and H. P. Koeffler.** 1988. p53 in chronic myelogenous leukemia. Study of mechanisms of differential expression. J. Exp. Med. **167**:873–886.

31a. **Lugo, T. G., and O. N. Witte.** 1989. The *BCR-ABL* oncogene transform Rat-1 cells and cooperates with v-*myc*. Mol. Cell. Biol. **9**:1263–1270.

32. **Maddon, P., D. Littman, M. Godfrey, D. Maddon, L. Chess, and R. Axel.** 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. Cell **42**:93–104.
33. **Martin, P. J., V. Najfeld, and P. J. Fialkow.** 1982. B-lymphoid cell involvement in chronic myelogenous leukemia: implications for the pathogenesis of the disease. Cancer Genet. Cytogenet. **6**:359–368.
34. **McLaughlin, J., E. Chianese, and O. N. Witte.** 1987. *In vitro* transformation of immature hematopoietic cells by the P210 *bcr/abl* oncogene product of the Philadelphia chromosome. Proc. Natl. Acad. Sci. USA **84**:6558–6562.
35. **Mes-Masson, A.-M., J. McLaughlin, G. Q. Daley, M. Paskind, and O. N. Witte.** 1986. Overlapping cDNA clones define the complete coding region for the $p210^{c\text{-}abl}$ gene product associated with chronic myelogenous leukemia cells containing the Philadelphia chromosome. Proc. Natl. Acad. Sci. USA **83**:9768–9772.
36. **Namen, A. E., S. Lupton, K. Hjerrild, J. Wignall, D. Y. Mochizuki, A. Schmierer, B. Mosley, C. J. March, D. Urdal, S. Gillis, D. Cosman, and R. G. Goodwin.** 1988. Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature (London) **333**:571–573.
37. **Nowell, P. C., and D. A. Hungerford.** 1960. A minute chromosome in human chronic granulocytic leukemia. Science **132**: 1497.
38. **Pierce, J. H., P. P. DiFiore, S. A. Aaronson, M. Potter, J. Humphrey, A. Scott, and J. N. Ihle.** 1985. Neoplastic transformation of mast cells by Abelson MuLV: abrogation of IL-3 dependence by nonautocrine mechanism. Cell **41**:685–693.
39. **Rennick, D., G. Yang, L. Gemmell, and F. Lee.** 1987. Control of hemopoiesis by a bone marrow stromal cell clone: lipopolysaccharide- and interleukin-1-inducible production of colony-stimulating factors. Blood **69**:682–691.
40. **Ribeiro, R. C., M. Abromowitch, S. C. Raimondi, S. B. Murphy, F. Behm, and D. L. Williams.** 1987. Clinical and biological hallmarks of the Philadelphia chromosome in childhood acute lymphoblastic leukemia. Blood **70**:948–953.
41. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase. I. J. Mol. Biol. **113**:237–251.
42. **Rogers, J., P. Early, C. Carter, K. Calame, M. Bond, L. Hood, and R. Wall.** 1980. Two mRNAs with different 3′ ends encode membrane-bound and secreted forms of immunoglobulin μ chain. Cell **20**:303–312.
43. **Rowley, J. D.** 1973. A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining. Nature (London) **243**:290–291.

44. **Saglio, G., A. Guerrasio, C. Ponzetto, A. Zaccaria, P. Testoni, B. Celso, G. R. Cambrin, A. Serra, L. Pegoraro, G. C. Avanzi, V. Attadia, M. Falda, and F. Gavosto.** 1988. Variability of the molecular defects corresponding to the presence of a Philadelphia chromosome in human hematologic malignancies. Blood **72**:1203–1208.
45. **Sandberg, A. A.** 1980. The chromosomes in human cancer and leukemia. Elsevier Scientific Publishing, Inc., New York.
46. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
47. **Schwartz, R. C., L. W. Stanton, S. C. Riley, K. B. Marcu, and O. N. Witte.** 1986. Synergism of v-*myc* and v-Ha-*ras* in the in vitro neoplastic progression of murine lymphoid cells. Mol. Cell. Biol. **6**:3221–3231.
48. **Secker-Walker, L. M., H. M. G. Cooke, C. A. Browett, J. D. Norton, E. Coustan-Smith, and A. V. Hoffbrand.** 1988. Variable Philadelphia breakpoints and potential lineage restrictions of bcr rearrangement in acute lymphoblastic leukemia. Blood **72**:784–791.
49. **Shen, F., Y. Saga, G. Litman, G. Freeman, J. Tung, H. Cantor, and E. Boyse.** 1985. Cloning of ly-5 cDNA. Proc. Natl. Acad. Sci. USA **82**:7360–7363.
50. **Shtivelman, E., B. Lifshitz, R. P. Gale, and E. Canaani.** 1985. Fused transcript of *abl* and *bcr* genes in chronic myeloid leukaemia. Nature (London) **315**:550–554.
51. **Strife, A., and B. Clarkson.** 1988. Biology of chronic myelogenous leukemia: is discordant maturation the primary defect? Semin. Hematol. **25**:1–19.
52. **Tachibana, N., S. C. Raimondi, S. J. Lauer, P. Sartain, and L. W. Dow.** 1987. Evidence for a multipotential stem cell disease in some childhood Philadelphia chromosome positive acute lymphoblastic leukemia. Blood **70**:1458–1466.
53. **Walker, L. C., T. S. Ganesan, S. Dhut, B. Gibbons, T. A. Lister, J. Rothband, and B. D. Young.** 1987. Novel chimeric protein expressed in Philadelphia positive acute lymphoblastic leukemia. Nature (London) **329**:851–853.
54. **Whitlock, C. C., D. Robertson, and O. N. Witte.** 1984. Murine B cell lymphopoiesis in long term culture. J. Immunol. Methods **67**:353–365.
55. **Whitlock, C. A., and O. N. Witte.** 1981. Abelson virus-infected cells can exhibit restricted in vitro growth and low oncogenic potential. J. Virol. **40**:577–584.
56. **Whitlock, C. A., and O. N. Witte.** 1982. Long term culture of B lymphocytes and their precursors from murine bone marrow. Proc. Natl. Acad. Sci. USA **79**:3608–3612.
57. **Witte, O. N., A. Dasgupta, and D. Baltimore.** 1980. The Abelson murine leukemia virus protein is phosphorylated *in vitro* to form phosphotyrosine. Nature (London) **283**:826–831.
58. **Young, J. C., and O. N. Witte.** 1988. Selective transformation of primitive lymphoid cells by the *BCR/ABL* oncogene expressed in long-term lymphoid or myeloid cultures. Mol. Cell. Biol. **8**:4079–4087.
59. **Ziegler, S. F., C. A. Whitlock, S. P. Goff, A. Gifford, and O. N. Witte.** 1981. Lethal effect of the Abelson murine leukemia virus transforming gene product. Cell **27**:477–486.