Clonal Evolution in a Myeloid Cell Line Transformed to Interleukin-3 Independent Growth by Retroviral Transduction and Expression of p210bcr/abl

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Current evidence suggests that the expression of the tyrosine kinase p210bcr/abl in chronic myelogenous leukemia (CML) may directly induce the initial phase of granulocytic hyperplasia. However, the dysregulation of additional genes appears to be required for transition to the acute leukemic phase, as inferred by the appearance of recurrent secondary cytogenetic abnormalities in the majority of patients. To determine whether the expression of p210bcr/abl alone is responsible for this genetic instability, we introduced and expressed the bcr/abl gene from a retroviral vector in a clone of the interleukin-3 (IL-3) dependent myeloblastic 32D C13(G) cell line. Clonal and polyclonal cells transformed to IL-3-independent growth were observed for a period extending up to 6 months for changes in the expression of p210bcr/abl, cell proliferation, inhibition by prostaglandin E1 (PGE1), forskolin, and cyclic adenosine monophosphate (cAMP) analogues, regulation of the cell cycle, and karyotype. Whereas the properties of control vector infected 32D C13(G)' cells remained stable over time, cells expressing p210bcr/abl were phenotypically unstable. In cells expressing p210bcr/abl, we observed selective modulation of p210bcr/abl mRNA and protein expression, evolution from partial to full abrogation of IL-3 dependence, reduced serum requirements, increased cell proliferation, decreased inhibition by PGE1; and cAMP analogues, and the appearance of new structural and numerical chromosomal abnormalities with successive cell passages. These results indicate that expression of p210bcr/abl can directly predispose 32D C13(G)' cells to genetic instability, promotes the emergence of clones with an increased proliferative advantage, and may represent an in vitro model suitable for the study of mechanisms underlying progression to the acute leukemic phase in CML.

CHRONIC MYELOGENOUS leukemia (CML) is a neoplastic disorder of pluripotent hematopoietic stem cells1 which is characterized cytogenetically by the presence of the Philadelphia chromosome (Ph),2 and at the molecular level by the translocation of c-abl sequences from chromosome 9 into the major breakpoint cluster region (M-bcr) of the bcr gene on chromosome 22.3 This results in the transcription of a novel 8.5-kb mRNA4,5 and the expression of a 210-Kd protein (p210bcr/abl) with constitutively active protein tyrosine kinase activity.6,7 The expression of p210bcr/abl alone appears to be sufficient to cause the granulocytic hyperplasia characteristic of the chronic phase in CML, as inferred by the induction of a similar disease in mice transplanted with syngeneic bone marrow cells expressing p210bcr/abl from a retroviral vector.8,9 However, secondary genetic events may be required for the evolution of chronic phase CML to acute blast crisis. Additional cytogenetic abnormalities including duplication of Ph, +8, (17q), +19, +21, −Y, or +Y are encountered in more than 80% of patients with CML in transition10 and the anti-oncogene p53 frequently is found to be inactivated.11,12 The mechanisms responsible for the genetic instability observed in CML are poorly understood, but presumably these can generate the clonal heterogeneity from which subclones with an increased proliferative advantage can emerge and come to predominate in the acute leukemic phase.

In a previous study involving retroviral mediated gene transfer and expression of p210bcr/abl in the interleukin-3 (IL-3) dependent myeloblastic 32D C13(G) cell line, we reported that infected cells were transformed to IL-3 independent growth by a nonautocrine mechanism, that granulocyte colony-stimulating factor (G-CSF) induced granulocytic differentiation was blocked, and that regulation of the cell cycle was altered resulting in an increased fraction of cells in G2/M.13 However, compared to cells infected with virus lacking the bcr/abl cDNA insert, polyclonally p210bcr/abl transformed cells appeared to undergo phenotypic changes with time, suggesting that p210bcr/abl expression could lead to clonal heterogeneity in vitro. In this report, we document clonal evolution of clonal and polyclonal p210bcr/abl transformed 32D C13(G)' cells with respect to gene expression, cell growth, regulation of the cell cycle, and karyotype within a 6-month period of observation after gene transfer. Our results indicate that p210bcr/abl transformed 32D C13(G)' cells are karyotypically unstable, develop a proliferative advantage in vitro over time, and appear to mimic the changes associated with the transition of the chronic phase of CML to the acute leukemic phase.

MATERIALS AND METHODS

Cell culture. The murine 32D C13(G) cell line is a subclone of 32D cells14 that originally was isolated by long-term in vitro cultivation of murine bone marrow cells, and was obtained from Dr G. Rovera (The Wistar Institute, Philadelphia, PA). This nontumorigenic IL-3 dependent myeloblastic cell line may be induced to terminal granulocytic differentiation with G-CSF15 or to undergo myelomonocytic differentiation in the presence of G-CSF and granulocyte-macrophage (GM)-CSF.16 When originally described,15 32D C13(G) cells had a normal karyotype. Examination of the clone that we obtained indicated that cells had become pseudodip-
light with the karyotype 39,XY,−2,−4,+der(2)t(2;4)(A1;A1) (described below). This subclone, which we have designated 32D C13(G)', is otherwise indistinguishable from the original cell line, has an absolute requirement for IL-3, and can be induced to terminally differentiate along the granulocytic lineage with G-CSF. Cells were maintained in α-MEM (GIBCO, Burlington, Ontario, Canada) containing 10% fetal bovine serum (FBS; GIBCO) at 37°C and 5% CO2. WEHI-3B cell17 conditioned medium (CM) was used as a source of crude mIL-318 as previously described, and filtered before use (0.22 μm/L; Nucleopore, Pleasanton, CA). Induction of granulocytic differentiation in cells treated with 2 ng/mL recombinant human (rh) G-CSF (Genetics Institute, Boston, MA) was determined by assay of dianisidine oxidation by myeloperoxidase in cell lysates as previously described.13

Vectors. The construction of pYNbcrlabl, a replication defective retroviral vector that expresses p210bcrlabl from the Moloney murine leukemia derived vector pYN,19 has been previously described.13 This construct expresses a bcr/abl cDNA insert from the 5' LTR promoter and the selectable gene neo, which confers resistance to the neomycin analogue G418 in mammalian cells, is coexpressed from an internal herpes simplex virus thymidine kinase (HSV tk) promoter (Fig 1A). Infectious virus was produced by transfection of pYN and pYNbcrlabl plasmids into the ecotropic ψ29 packaging cell line followed by secondary infection of modified amphotropic PA31726 cells as described earlier13 or by transient expression of vectors in the ecotropic GP+E-8625 packaging cell line. Transfections were performed by resuspending 5 × 106 cells in 0.4 mL of 10% FBS α-MEM containing 40 μg of circular vector plasmid, followed by electroperoration at 250 V and 960 microfarads (ie, 7/2 of 15 to 25 milliseconds) at room temperature (Gene Pulser with capacitance extender; Bio-Rad, Richmond, CA).

Gene transfer. Polyclonal infected populations of 32D C13(G)' cells were obtained by resuspending 1 × 106 cells in 5 mL of 0.22 μmol/L filtered culture supernatants from pYN or pYNbcrlabl transfected packaging cells with 4 μg/mL polybrene (Sigma, St Louis, MO) and 10% WEHI-3B CM. Beginning 24 hours after infection, cells were selected for 10 days with 600 μg/mL of G418 (GIBCO). Whereas G418 resistant pYN infected cells all died within 48 hours of IL-3 withdrawal and required WEHI-3B CM for long-term growth, similar to uninfected parental 32D C13(G)' cells, pYNbcrlabl infected cells continued to proliferate in the absence of IL-3. To study the evolution of intraclonal heterogeneity, subclones of p210bcrlabl expressing cells derived from a single infected cell were isolated shortly after infection. Clones were obtained by infecting 32D C13(G)' cells with low titer pYNbcrlabl virus (ie, diluted to approximately 1 × 104 cfu/mL) to decrease the number of independently infected target cells, and these were subcloned by limiting dilution in 96 microwell plates in the absence of WEHI-3B CM after 10 days of selection with G418. Clones were subsequently expanded and subjected to 5' LTR BamHI RFLP analysis to identify those that carried the same RFLP and thus could be shown to have originated from a single common infected cell (see below).

Southern and Northern blotting analysis. Genomic DNA was isolated using standard methods from pYNbcrlabl virus infected clones that had been isolated by limiting dilution. Samples were digested with BamHI, electrophoresed through 0.8% agarose, blotted onto nylon membranes (Nytran; Schleicher & Schuell, Keene, NH) and, hybridized to a neo probe labeled with [α-32P]-dCTP to a specific activity of 1 to 2 × 106 cpm/μg using a commercial nick-translation kit (Bethesda Research Laboratories [BRL], Burlington, Ontario, Canada). Conditions for prehybridization and hybridization were as recommended by the manufacturer (Schleicher & Schuell). The final wash was with 0.1% SSC, 0.5% sodium dodecyl sulfate (SDS) for 30 minutes at 65°C before exposure to Kodak X-Omat film with one intensifying screen at −70°C. The restriction endonuclease BamHI cuts the pYNbcrlabl vector polylinker immediately 5' to the HSV-Tk-neo insert. No BamHI sites are located 3' to this site in the vector and thus the next 3' BamHI site is found in the flanking genomic DNA of the target cell (Fig 1A). Because retroviral integration occurs randomly in the genome, BamHI digestion creates RFLPs hybridizing to the neo gene that are unique for each 3'-LTR integration. This approach was used to identify the clonal origin of infected cells.

Northern blot analysis was performed using total cell RNA isolated by acid guanidium thiocyanate-phenol-chloroform extraction21; 10 μg of RNA was electrophoresed in a 1% agarose-formaldehyde gel, transferred to Nytran, and hybridized to the neo probe as described above. In pYN virus infected cells, the neo gene

Fig 1. (A) Structure of the replication defective retroviral vector pYNbcrlabl showing the origins of RNA transcripts from the 5'-LTR and HSV tk promoters, the most 3' BamHI restriction endonuclease site in the vector, the next 3' BamHI site in flanking genomic DNA, and the neo gene probe. (B) Southern blot of genomic DNA digested with BamHI, isolated from uninfected 32D C13(G)' cells (32D) and, from 14 clones containing an identical 3.8-kb 3'-LTR BamHI RFLP, indicating that they all arose from a single infected cell containing one copy of the bcr/abl virus. Differences in band intensity are caused by unequal loading of DNA.
probe hybridizes to 3.5- and 2.1-kb mRNAs derived from the 5' LTR and the HSV tk promoters, respectively. A longer 10.5-kb message containing the bcr/abl sequences is expressed from the 5' LTR promoter in pYNbcr/abl virus infected cells in addition to the 2.1-kb HSV tk driven neo transcript.13

Analysis of phosphotyrosyl-containing proteins. One million cells were washed once with ice-cold phosphate-buffered saline (PBS) in a microfuge tube. Cell pellets were rapidly resuspended in 40 μL of lysis buffer (20 mmol/L Tris-HCl pH 8.0, 132 mmol/L NaCl, 2 mmol/L EDTA, 100 μmol/L vanadate, 1 mmol/L PMFS, 1% Triton-X-100, and 10% glycerol) on ice. Forty microliters of 2× Laemmli sample buffer (120 mmol/L Tris pH 6.8, 2 mmol urea, 100 mmol/L DTT, 10% vol/vol glycerol and 0.001% wt/vol bromophenol blue) was immediately added with vortexing and samples were boiled for 3 minutes. Fifty microliters of sample and molecular weight markers (Sigma) were electrophoresed on a vertical 4% to 12% gradient Laemmli SDS-polyacrylamide slab gel. After electrophoresis, proteins were electrophoretically transferred onto NitroScreen West membrane, and nonspecific binding sites were saturated by a Tris-HCl pH 7.5, 0.9% NaCl), containing 3% bovine serum albumin (BSA), for 1 to 2 hours at room temperature. After extensive washing in TTBS, membranes were subcloned by limiting dilution in 96-well microtiter plates in the absence of WEHI-3B CM. Approximately one third of G418 resistant cells plated emerged as auton-

RESULTS

Isolation of subclones from a single cell expressing p210bcr/abl. In previous studies, we examined the short-term effects of p210bcr/abl expression in 32D Cl3(G)" cells polyclonally infected with bcr/abl virus.13 However, when these cells were carried for longer periods of time various parameters, including adherence to tissue culture flasks, cell growth, and the distribution of cellular DNA content, appeared to change with time, whereas the properties of control (ie, pYN) vector infected cells remained stable. To determine whether this apparent instability was a direct consequence of p210bcr/abl expression or was simply caused by the selection of independently infected clones from a complex mixture, we observed the effects of p210bcr/abl in individual clones over several months. To minimize any variation that might result from examining independently infected clones, such as altered gene expression caused by retroviral position effects, we elected to study subclones derived from a single infected cell shortly after infection.

32D C13(G)" cells infected with low-titer bcr/abl virus and selected with G418 for 10 days in 10% WEHI-3B CM were subcloned by limiting dilution in 96-well microtiter plates in the absence of WEHI-3B CM. Approximately one third of G418 resistant cells plated emerged as autonomously growing clones and these were examined for the presence of clonal retroviral integration sites by Southern blotting. Fourteen clones were found that contained an identical 3.8-kb 3' LTR integration site RFLP using BamHI, identifying these as subclones of a single infected cell containing one copy of the bcr/abl retrovirus (Fig 1B). These results were confirmed by the presence of unique 3' LTR integration site RFLPs of 24 kb and 5.3 kb with XhoI and BglII, respectively (data not shown). Supernatants from these clones were unable to maintain the growth of parental cells in the absence of IL-3 (data not shown). Also, all clones stimulated with rhG-CSF (2 ng/mL) for 10 days had levels of myeloperoxidase equal to unstimulated cells (ie, diaminodiphenyl sulfone oxidation rate ≦0.2 mmol/min/10⁶ cells), whereas in control pYN infected cells, myeloperoxidase levels increased to approximately 6.0 to 7.0 mmol/min/10⁶ cells (data not shown). These results indicated that the growth of the subclones isolated was stimulated by a nonautocrine mechanism and that induction of granulocytic differentiation by rhG-CSF was blocked, as previously reported in polyclonally transformed cells.13 These subclones were studied longitudinally in parallel with polyclonal infected 32D C13(G)" cells infected with either high-titer bcr/abl or control pYN virus.

Intraclonal heterogeneity of bcr/abl expression. Analysis of bcr/abl mRNA (Fig 2) and phosphotyrosyl protein (Fig 3) expression showed marked intraclonal heterogeneity within a relatively short time period after infection. Whereas the expression of the viral neo gene from the internal tk promoter remained relatively constant, the amount of full-length viral mRNA containing the bcr/abl cDNA expressed from the 5' LTR promoter varied from nearly undetectable levels (ie, clones LE4, SA9, SB12) to hybridization signals stronger than those of the shorter neo transcript (ie, clones LE10, MB2, MD1). This suggests that the expression of the bcr/abl message was selectively modulated (ie, compared with neo), by altered regulation of transcription from the 5'-LTR promoter, mRNA stability, or other factors. The expression of phosphotyrosyl containing proteins including p210bcr/abl (Fig 3) was similarly
highly variable within subclones. The relative levels of bcr/abl mRNA and p210bcr/abl appeared to correlate poorly, implicating a divergence in the efficiency of mRNA translation, protein stability, control of phosphorylation by phosphatases or by other mechanisms amongst the different clones.

**Cell proliferation.** 32D C13(G)' cells polyclonally infected with bcr/abl virus initially divide slowly on withdrawal from IL-3, but subsequently increased their rate of proliferation to that of maximally stimulated control infected cells (in the absence of serum) or greater than control in the presence of serum 3 to 4 weeks after infection. Subsequently, polyclonal p210bcr/abl expressing cells appeared to increase their proliferative rate further. As shown in Fig 4, the activity of individual p210bcr/abl expressing subclones increased from 52 to 108 days after infection in comparison with maximally stimulated control pYN infected cells. Three of the nine clones examined 52 days after infection could be stimulated further with WEHI-3B CM (ie, clones LG7, MD1, and MG10), demonstrating a residual capacity to respond to IL-3. When these were re-examined 108 days after infection, the addition of WEHI-3B CM no longer had any effect, consistent with the complete abrogation of IL-3 dependence. These results suggested that the complete functional substitution of IL-3 dependence by p210bcr/abl occurred step-wise. We have never detected residual IL-3 responsiveness in polyclonally infected cells even when they
Fig 4. MTT cell proliferation assay of control (PYN) infected and p210bcr/abl expressing 32D CI3(G)' cells 52 days (top panel) and 108 days (bottom panel) after infection. Cells were plated at an initial density of 1 x 10^4/well ± 10% WEHI-3B CM and were assayed 48 hours later using the MTT procedure. Results are expressed as the mean of four quadruplicate measurements ± 1 SD. Three clones* (LG7, MD1, and MG10) showed a significant (P < .05) residual responsiveness to WEHI-3B CM at 52 days, but not at 108 days, postinfection.

were examined as early as 3 weeks after infection, suggesting that cells which have complete abrogation of IL-3 dependence also have a selective growth advantage and can rapidly predominate in polyclonal cultures.

Inhibition of cell growth. We next examined the response of cells to agents that can modulate the activity of the adenyl cyclase pathway, which is an important inhibitory pathway of myeloid cell growth. PGE1 binds to a membrane receptor that is coupled to and activates adenyl cyclase in myeloid cells.26 As shown in Fig 5, PGE1 did not inhibit the growth of any transformed clones except for one (LE10). Parallel measurements of the inhibition mediated by forskolin, a direct agonist of adenyl cyclase, indicated that some clones were relatively insensitive (ie, MD7, MG10, SA9, and SB12), moderately sensitive (ie, LG9, MB2, MD1, and MG5), or had wild-type sensitivity (ie, LE4, LE10, LG7, and MAS). These results suggested that the loss of PGE1-mediated inhibition of cell growth in some clones developed from a failure of PGE1 to stimulate adenyl cyclase (ie, in forskolin-sensitive clones), whereas in others this could be accounted for by a relative resistance to forskolin. To determine whether the heterogeneous loss of forskolin response occurred at the level of adenyl cyclase or further downstream, we measured the inhibition of cells treated with analogues of cAMP. The inhibition mediated by dibutyryl-cAMP (Fig 5) or 8-bromo-cAMP (not shown) correlated well (ie, r = .90) with that of forskolin in individual clones, indicating that variation in the insensitivity of different clones to cAMP could account for failure of forskolin to inhibit cell growth. Four weeks later, the LE10 clone had also become insensitive to PGE1 and acquired intermediate sensitivity to forskolin, suggesting that the loss of growth inhibition occurs stepwise either by failure of PGE1 to activate adenyl cyclase (early) and/or from an acquired insensitivity to cAMP.

Analysis of cellular DNA content. In the first 4 weeks after polyclonal bcr/abl virus infection, 40% to 50% of cells have a G2/M DNA content as assessed by flow cytometry, in contrast to ≤5% in parental or control vector infected 32D CI3(G)' cells.13 Over a period of 2 to 3 months we subsequently observed a progressive reduction of the percentage of cells with G2/M DNA content, compensated by an increase in both G0/G1 and S phase DNA content. Flow cytometric analysis of nuclear DNA content of p210bcr/abl expressing subclones 70 days postinfection (Fig 6, top panel), showed that the fraction of p210bcr/abl transformed cells in S phase was much greater than that of control infected parental cells growing logarithmically in the presence of WEHI-3B CM. All 13 clones examined also had an increased fraction of G2/M DNA content that was greatest in clones MB2 and MD7. When re-examined 182 days postinfection, we noted a general reduction in the G2/M fraction that was compensated by fractional increases of G0/G1 or S (Fig 6, bottom panel). These results extend our earlier observations13 and demonstrate that the accumulation of cells with G2/M DNA content induced by the expression of p210bcr/abl in polyclonally infected 32D CI3(G)' cells is reversed by a mechanism of clonal adaptation.

Fig 5. Relationship of the response of cell clones to PGE1 (top panel) or to dibutyryl-cAMP (bottom panel), with forskolin assayed by the MTT method. Values are given as the mean fractional growth (Fa) of four independent values ± 1 SD, relative to untreated controls for each cell line.
In 2 of 11 p210bcr/abl expressing subclones (ie, MD7 and SA9), we observed the transient appearance of hyperdiploid cell populations, and an additional clone (ie, SA8) maintained a stable near-tetraploid DNA content (Fig 7). In MD7 and SA9, the hyperdiploid cell population was clearly derived from the original clone and must have acquired a growth advantage to become detectable. This was confirmed directly in the SA9 clone by observing the effect of serum deprivation followed by serum rescue over a period of 48 hours (Fig 8). DNA histograms were analyzed with separate integration of the diploid and aneuploid peaks in the population. In previous studies we had determined that transformed cells deprived of serum accumulated in G0/G1. As shown in Fig 9, the ratio of the diploid to the aneuploid G0/G1 peaks began to increase 4 hours after serum withdrawal and peaked after 8 hours, indicating that diploid cells arrested in G0/G1 sooner than did the aneuploid cells. Twenty-four hours after serum withdrawal, 10% FBS was added and resulted in a sharp decrease of the G0/G1 ratio over 2 hours, indicating that diploid cells were slower to exit from G0/G1 than were the aneuploid cells. However, this decreased serum dependence of the hyperdiploid cell population did not ensure its dominance long-term because repeat analysis 182 days postinfection showed that SA9 had returned to a diploid DNA content (Fig 6). This suggests that another event had occurred in the diploid cell population which conferred a growth advantage sufficient to completely overtake the hyperdiploid cell population over a period of 60 days.

Karyotype analysis. The appearance of aneuploid cell DNA content prompted us to perform karyotype analysis and examine whether new structural chromosomal abnormalities might develop in cells expressing p210bcr/abl (Table 1). Parental uninfected and pYN infected 32D C13(G)- cells both had diploid DNA content and a stable 39,XY,-2,-4,+der(2)t(2;4)(A1;A1) karyotype (Fig 10A). Examination of a polyclonal population of 32D C13(G)- cells expressing p210bcr/abl 2 months after infection showed a complex assortment of new chromosomal translocations and double minute chromosomes, indicating that p210bcr/...
When the series of subclones derived from a single bcr/abl infected cell was examined, we found the parental t(2;4) marker chromosome to be absent in 3 of 13 clones (i.e., LE10, SA9, SB12) or missing in some cells in another 4 clones (i.e., LE4, LG9, MD7, MG10). In addition, all clones contained a new translocation, t(4;12), suggesting that this marker chromosome was created before subcloning by limiting dilution 10 days after infection. A second new marker chromosome, t(2;17), was found in three clones (MD7, SA9, SB12) and 10 of 13 clones also had additional unique clonal marker chromosomes. The SA9 clone that contained both the t(4;12) and t(2;17) marker chromosomes is shown in Fig 10B. Six of the subclones (LE4, LG9, MD1, MD7, MG10, SB12) consisted of two or three karyotypically distinct clonal population of cells, indicating that karyotypic evolution was also frequent after individual cells were isolated by limiting dilution. Approximately 2% of polyclonally infected cells were also found to contain the t(4;12) marker chromosome, suggesting that this represents a nonrandom chromosomal translocation. It remains to be determined whether the frequency of the t(4;12) marker chromosome in polyclonally infected cells will increase in the population with successive cell passages. Nevertheless, these results indicate that the expression of p210bcr/abl can induce a high frequency of structural chromosomal abnormalities in 32D C13(G) cells.

**DISCUSSION**

The natural evolution of chronic myelogenous leukemia (CML) from a chronic to an acute leukemic phase is the main determinant of patient survival. The molecular mechanisms underlying this progression are poorly understood, although the secondary activation of N-ras27-29 and c-myc30 genes in a small percentage of patients, the functional inactivation of the anti-oncogene p53 in approximately 30% of patients,11,29,31,32 or loss of the retinoblastoma gene product in CML megakaryoblastic crisis33 may be contributing factors. Even though duplication of the Philadelphia chromosome is a frequent event in CML blast crisis,30 bcr/abl expression does not appear to be consistently increased,33 although there are exceptions.34 The deletion of bcr/abl in CML blast crisis has been described in several patients,35,37 suggesting that the expression of p210bcr/abl may precede acquired secondary genetic or epigenetic events that may independently maintain dysregulated hematopoietic cell growth. Although genetic instability in CML is generally appreciated to play a central role in the pathogenesis of CML, the number of studies that have sought to develop model systems to facilitate its study have been limited. Retroviral mediated transfer and expression of p210bcr/abl in murine hematopoietic cells established in long-term bone marrow culture has been shown to result in the outgrowth of immature B-lymphoid cells which, with time, demonstrate an increased plating efficiency in agar suspensions and increased tumor induction in syngeneic mice.36 Similar experiments performed with Abelson murine leukemia virus (A-MuLV) infected mouse bone marrow cells also indicated that development of a tumorigenic potential requires multiple steps.39

In this report, we demonstrate that the expression of p210bcr/abl in a murine IL-3 dependent myeloid cell line results in phenotypic progression that mimics many of those associated with transition of chronic phase CML to the acute leukemic phase. Expression of the tyrosine kinases p210bcr/abl,31-33 v-abl,40 or v-src41 in the 32D C13(G) cell line inhibits G-CSF induced terminal granulocytic differentiation and mimics the maturational arrest associated with CML in transition. It is interesting to observe that expression of phosphotyrosyl protein phosphatase (PTPP) increases with granulocytic differentiation41 and indeed may be essential for the attenuation of cell proliferative capacity that accompanies cell maturation. Although the expression of bcr/abl mRNA may not be significantly altered between chronic and blast phase CML, the detection of p210bcr/abl associated tyrosine phosphorylation is more difficult in mature granulocytes because of elevated levels of PTPP.42 At present, it is not known whether reduced PTPP in blast phase CML is caused by failure of cell maturation or whether an acquired defect in phosphatase expression contributes to cell differentiation. Overexpression

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**Table 1. Karyotype Analysis of Parental Cell (32D) and p210bcr/abl Expressing Subclones**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Karyotype</th>
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<tbody>
<tr>
<td>32D</td>
<td>39,XY,-2,-4,+der(2)(2;4)(A1;A1)</td>
</tr>
<tr>
<td>LE4</td>
<td>39,XY,-4,-12,+der(12)(4;12)(E1;A1;A1)</td>
</tr>
<tr>
<td>LE10</td>
<td>39,XY,-4,-12,+der(12)(4;12)(E1;A1;A1),+mar(18)</td>
</tr>
<tr>
<td>LG7</td>
<td>40,XY,-4,-12,+der(2)(2;4)(A1;A1),-4,-12,+der(12)(4;12)</td>
</tr>
<tr>
<td>LG9</td>
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</tr>
<tr>
<td>MB2</td>
<td>39,XY,-4,-12,+der(12)(4;12)(E1;A1),+mar(16)</td>
</tr>
<tr>
<td>MD1</td>
<td>40,XY,-4,-12,+der(12)(4;12)(E1;A1),+mar(16)+mar(16)</td>
</tr>
<tr>
<td>MD7</td>
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<td>MG5</td>
<td>39,XY,-2,-4,+der(2)(2;4)(A1;A1),-4,-12,+der(12)(4;12)</td>
</tr>
<tr>
<td>MG10</td>
<td>40,XY,-4,-12,+der(12)(4;12)(E1;A1),+mar(16)+mar(16)</td>
</tr>
<tr>
<td>SA8</td>
<td>Near tetraploid, XY,der(2)(2;4)(A1;A1),der(12)(4;12)</td>
</tr>
<tr>
<td>SA9</td>
<td>39,XY,-2,+der(2)(2;17)(A1;A1),-4,-12,+der(12)(4;12)</td>
</tr>
<tr>
<td>SB12</td>
<td>39,XY,-2,+der(2)(2;17)(A1;A1),-4,-12,+der(12)(4;12)</td>
</tr>
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Thirty metaphase cells were examined for each clone.
of PTPP can inhibit cell transformation by tyrosine kinases and it is possible that the converse situation, such as the over-expression of p210bcr/abl in 32D C13(G') cells, can overwhelm cellular PTPP and block granulocytic differentiation.

The complete transformation of 32D C13(G') cells by p210bcr/abl was shown to occur by a multi-step process that could not easily be related to the intraclonal heterogeneity of bcr/abl expression which we observed. The progressive increase in the proliferative capacity of p210bcr/abl expressing cells over time was particularly striking. Several determinants of 32D C13(G') cell growth were monitored and all were altered over time in a way that might be expected to enhance cell growth. Some p210bcr/abl expressing clones were shown to retain a minimal capacity to respond to IL-3, which was subsequently lost, suggesting that full independence from IL-3 requires other cellular adaptations. Detailed examination of the SA9 clone also associated the role of decreased serum dependence with a proliferative advantage. The expression of p210bcr/abl in 32D C13(G') cells was shown to confer resistance to PGE1-mediated inhibition of cell growth and is similar to the PGE1-resistant growth described in CML derived CFU-GM cells. Most of the resistance observed could be attributed to the decreased effectiveness of cAMP analogues to inhibit cell growth, although in some instances this appeared to occur upstream at the level of the coupling of PGE1 to the adenyl cyclase pathway. One p210bcr/abl expressing clone (i.e., LE10) initially was observed to have wild-type sensitivity and to develop resistance later, suggesting that this phenotype is not an immediate consequence of p210bcr/abl expression. Further adaptation of the cell population could have resulted from clonal selection. Lastly, the expression of p210bcr/abl was also noted to result in an accumulation of cells in G2/M, which presumably represents a partial block of cell cycle progression. That the fraction of cells in G2/M tended to decrease with time in association with increased cell proliferation suggests that cells may have gained a proliferative advantage by bypassing this block.

Although the exact mechanisms underlying the phenotypic progression outlined above remain unknown, we have provided evidence that these are associated with the induction of genetic instability in 32D C13(G') cells by p210bcr/abl. Both numerical and structural chromosomal abnormalities, the hallmark of transformed neoplastic cells, were noted to occur at a high frequency. It is of interest that in lymphoblastic leukemia/lymphoma cells which develop in transgenic mice bearing a transgene that expresses p190bcr/abl, a high frequency of chromosomal abnormalities was observed that involved chromosomes 12, 14, and 17 preferentially. Thus, it is striking that we observed a similar preference for the development of abnormalities involving chromosomes 12 and 17 in 32D C13(G') cells transformed by retrovirus expressing p210bcr/abl, suggesting that the in vitro evolution of karyotype abnormalities may approximate that which develops in vivo. Clonal chromosomal translocations as we have observed in p210bcr/abl transformed 32D C13(G') cells are relatively rare occurrences in cultured cells unless defects in DNA repair are present. Whether p210bcr/abl can directly interfere with DNA repair mechanisms is an intriguing possibility that needs to be explored. The p210bcr/abl induced accumulation of cells in G2/M that we have observed may be consistent with this possibility because this is characteristically observed in cells undergoing DNA repair after ionizing radiation induced DNA damage or in patients with hereditary deficiency of...
DNA repair as in Fanconi's anemia.\textsuperscript{40} Hyperphosphorylation of the cell cycle regulating p34cdc2 kinase by p21Obr/ abl also could conceivably result in the accumulation of cells in G$_2$/M because dephosphorylation of Tyr$_{15}$ is essential for entry into mitosis.\textsuperscript{40}

Karyotypic instability may not simply be the consequence of neoplastic cellular transformation but may be a primary event in cellular transformation and tumor progression. The expression of oncogenic p21ras from a dexamethasone inducible vector in NIH 3T3 fibroblasts was recently shown to reversibly cause lagging of chromosome movements in prometaphase, thus increasing the chance of asymmetrical anaphase and aneuploidy.\textsuperscript{50} This effect could be shown to occur before full cell transformation, supporting an initiating and causative role for karyotypic instability in tumor progression.\textsuperscript{50} The promotion of cell transformation and transformation by various oncogenes are not necessarily equivalent and in some instances have been partially dissociated.\textsuperscript{61} Consequently, there is a possibility that the mechanisms underlying transformation to CML blast crisis can be identified and specifically targeted in the future.

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