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Expression of cellular oncogenes in primary cells from human acute leukemias

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ABSTRACT **The structure and the expression of 11 cellular oncogenes (protooncogenes) were analyzed in primary cells from 20 acute lymphocytic (ALL) and 31 acute myelogenous (AML) leukemia patients. Neoplastic cells, obtained prior to initiation of therapy, were purified and classified, on the basis of both surface antigen pattern and morphology, into pre-B, B, and T ALL and M1–M5 AML. RNA was extracted and analyzed for expression of cellular oncogenes coding for nuclear proteins (c-*myc*, c-*myb*, c-*fos*), the β-chain of platelet-derived growth factor (c-*sis*), growth factor receptors or related proteins (c-*src*, c-*abl*, c-*fes*, c-*erbB*), or putative intermediate transducers of mitogenic signals (c-Ha-*ras*, c-Ki-*ras*, c-N-*ras*). Quantitative analysis of total RNA was carried out by dot blot hybridization to specific cDNA or genomic probes. Number and size of transcripts were evaluated by blot hybridization of electrophoretically fractionated poly(A)$^+$ RNA. Expression of c-*myc* and c-*myb* was detected in all leukemic cells at variable levels and was characterized by well-defined patterns within ALL subtypes. Conversely, significant levels of c-*fos* transcripts were detected only in myelomonocytic (M4) and monocytic (M5) leukemias. Among the "*src*-family," c-*fes* was expressed more in AML than ALL, and c-*abl* was expressed at variable but not elevated levels in all leukemia types. c-Ha-*ras* was uniformly expressed at low levels, as in non-neoplastic cells. c-Ki-*ras* transcription was detected only in T ALL; N-ras expression was barely demonstrable. The structure of these protooncogenes was not grossly modified, as evaluated by Southern analysis, except for c-*myc* rearrangement in B ALL. These studies indicate that cellular oncogene expression in specific subtypes of leukemic cells may relate to either the proliferative activity (c-*myc*, c-*myb*) or the differentiation state (c-*fos*) of the cells, or possibly to expression of receptors for putative hemopoiesis-related growth factors (c-*fes*, c-*abl*). Our data provide a basis for in-depth analysis of protooncogene expression in normal and neoplastic hemopoiesis.**

The cellular homologues of >20 acutely transforming viral oncogenes have been cloned and mapped on human chromosomes (1–5). These genes, transcribed in a variety of cells and tissues, admittedly play a key role in the regulation of normal cellular proliferation and/or differentiation (1, 4, 5). At least some of them exert their action at the level of different compartments along the mitogenic pathway—i.e., growth factors, membrane receptors, proteins transducing mitogenic signals to the nucleus, and nuclear proteins (6–9).

Alterations of cellular oncogene expression have been reported in numerous neoplasias, particularly of the hemolymphopoietic system (reviewed in refs. 1, 4, 5, 10, and 11). However, a cause–effect relationship between cellular oncogene abnormalities and oncogenesis is still not conclusively established. In this regard, most observations have been made on cell lines, which may not exactly reproduce the genotype and phenotype of the primary neoplastic clone(s) (12). On the other hand, comprehensive studies on cellular oncogene expression in primary leukemic cells have not been reported so far, and the limited data available have led to contradictory results (13–17).

We report a systematic study on the expression of 11 cellular oncogenes in primary cells from 51 acute lymphocytic (ALL) or myelogenous (AML) leukemia patients. Cells were obtained before initiation of therapy and were classified according to the presently accepted immunologic and morphologic criteria. We show that (*i*) c-*myc* and c-*myb* are consistently expressed in acute leukemias, with characteristic patterns within ALL subtypes, whereas c-*fos* is selectively expressed in M4–M5 AML; (*ii*) c-*fes* transcripts are more abundant in AML than in ALL, c-*abl* is variably expressed at low levels, and c-*src* and c-*erbB* transcripts are not detectable; (*iii*) expression of c-Ha-*ras* and N-*ras* is low or undetectable in all acute leukemias, whereas that of c-Ki-*ras* is observed only in T-cell ALL; (*iv*) the c-*sis* gene is apparently never expressed in AML or ALL.

MATERIALS AND METHODS

Patients and Cells. We have selected 51 hematology–oncology patients with acute leukemia of either myelogenous or lymphocytic type. Differential diagnosis of ALL as pre-B, B, or T type and of AML as M1–M5 type was carried out both by morphological evaluation of bone marrow smears according to the French–American–British (FAB) criteria (18) and by immunological analysis of leukemic cells with a panel of commercially available monoclonal antibodies (Ortho Diagnostic; Coulter; Becton Dickinson). Leukemic cells, obtained from peripheral blood or bone marrow aspirates immediately after diagnosis and before initiation of therapy, were enriched up to >90% by centrifugation on standard Ficoll-Paque cushions. Karyotype analysis of all leukemic samples was carried out by standard trypsin–Giemsa banding techniques.

Probes. The following cellular or viral oncogene probes were used: a 1.0-kilobase (kb) cDNA sequence (pRyc 7.4) of human c-*myc* (19); a 2.0-kb *Eco*RI–*Eco*RI genomic fragment (pF-8) containing the 3′ region of c-*myb* (20); a 1.3-kb *Bgl* II–*Pvu* II fragment containing most of v-*fos* (21); a 3.8-kb *Bam*HI–*Bam*HI sequence (p26) containing most of human c-*fes* (22); a 0.65-kb *Eco*RI–*Bam*HI fragment containing the 5′ exon of human c-*abl*; a 2.4-kb cDNA sequence (pE7) of the human epidermal growth factor receptor (c-*erbB*) gene (23); a 0.8-kb *Pvu* II–*Pvu* II fragment containing the 3′ portion of v-*src*; a 1.7-kb *Bam*HI–*Bam*HI sequence (pL335) containing

Abbreviations: FAB, French–American–British classification criteria; ALL, acute lymphocytic leukemia(s); AML, acute myelogenous leukemia(s); CML, chronic myelogenous leukemia(s); kb, kilobase(s).

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the 3′ exon of human c-*sis* (24); a 6.6-kb *Bam*HI–*Bam*HI fragment (pT24-C2) containing human c-Ha-*ras1* (25); a 1.0-kb *Hin*cII–*Hin*cII sequence (HiHi3) containing part of the v-Ki-*ras* (26); a 0.57-kb cDNA sequence (p6al) of human N-*ras* (27).

The human genomic immunoglobulin gene probes were the *Eco*RI–*Eco*RI fragments containing the joining (J_H) region of the heavy chain locus and the coding portion of the μ-chain constant region (C_μ) gene (28).

RNA Analysis. Total cellular RNA was extracted from $\approx 10^8$ cells by the guanidinium thiocyanate or guanidine·HCl technique (29), dotted (8–0.5 μg) onto nitrocellulose filters (BA-85, Schleicher & Schuell), and hybridized to 10^7 cpm of probe labeled by nick-translation to a specific activity of 3–7 $\times 10^8$ dpm/μg (30). After washing under stringent conditions [65°C; 0.1× SSC for homologous probes, 0.3× SSC for viral probes (1× SSC is 0.15 *M* NaCl/15 mM sodium citrate)], filters were exposed for 1–3 days at −70°C to Kodak SO-282 x-ray films. Expression of cellular oncogenes was evaluated by densitometric comparison with standard dots of 4 μg of human rRNA containing various amounts (usually 1–250 pg) of the human cDNA or genomic probe. Appropriate conversion factors were formulated for each probe, thus accounting for both the proportion of transcribed sequences, in the case of genomic probes, and their size as compared to full-length mRNA, in the case of genomic and cDNA probes (see ref. 31). The final data were expressed as pg of mRNA per μg of total RNA dotted onto the filters. In experiments with viral oncogene probes, the data were necessarily expressed in arbitrary units.

Poly(A)$^+$ RNAs obtained by one passage on oligo(dT)-cellulose columns (T-3 type, Collaborative Research, Waltham, MA) (32) were electrophoresed in 5-μg aliquots in formaldehyde-containing 0.8–1.2% agarose gels, transferred onto nitrocellulose filters by capillary blotting (30), and then hybridized, washed, and exposed as for dot blots.

DNA Analysis. High molecular weight DNA was extracted from $\approx 10^8$ cells (33), digested with restriction endonucleases, electrophoresed in 1% agarose gels, transferred onto nitrocellulose filters, and hybridized to 2×10^7 cpm of radiolabeled probe as described (32).

RESULTS

Classification of Leukemic Cells. Twenty ALL were classified as follows: one B-cell precursor ALL [HLA-DR$^+$, common ALL antigen (CALLA)-negative, immunoglobulin heavy-chain (IgH) gene rearranged], eight pre-B ALL (HLA-DR$^+$, CALLA$^+$, BA-1$^+$, B-2$^+$, one or both IgH genes rearranged), three B ALL [HLA-DR$^+$, surface Ig$^+$, t(8;14) chromosome marker], eight T ALL (erythrocyte-rosetting; subclassified according to reactivity to monoclonal antibodies OKT3, -T4, -T6, -T8, -T10, -T11, anti-Leu-1, and anti-Leu-9). Thirty-one AML were classified according to FAB classification (18) as follows: three M1, six M2 (myeloblastic), five M3 (promyelocytic), nine M4 (myelomonocytic), and eight M5 (monocytic). All M3 AML showed the t(15;17) chromosome marker.

Expression of Cellular Oncogenes c-*myc*, c-*myb*, and c-*fos*. The c-*myc* gene was transcribed at variable levels in all leukemic cells (Figs. 1 and 2), to give two mRNAs of 2.2 and 2.4 kb (Fig. 3). In pre-B ALL, the expression was low (2.9 ± 1.3 pg/μg of total RNA, mean ± SEM); in T ALL, slightly higher (8.3 ± 3.4 pg); and in B ALL, markedly more elevated (61 ± 24 pg). For comparison, the level in the HL-60 line, which carries >20 copies of the c-*myc* gene (34, 35), was 107

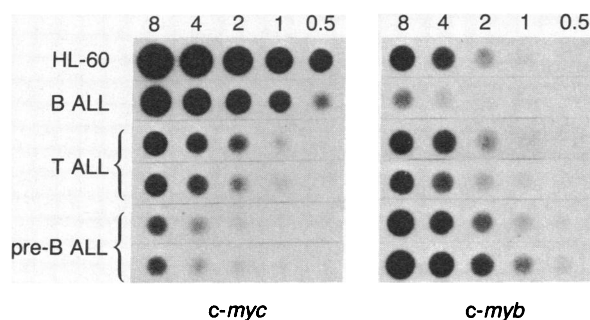

FIG. 1. Expression of c-*myc* (*Left*) and c-*myb* (*Right*) in the promyelocytic cell line HL-60 and in primary cells from representative patients with T-, pre-B-, and B-cell ALL. Total RNA extracted from purified blast cells was dotted (8 to 0.5 μg) onto nitrocellulose paper and hybridized to human c-*myc* cDNA and c-*myb* genomic probes. OD values were normalized between different filters as compared to internal positive controls, converted into absolute values (pg of transcripts per μg of total RNA), and plotted in Fig. 2.

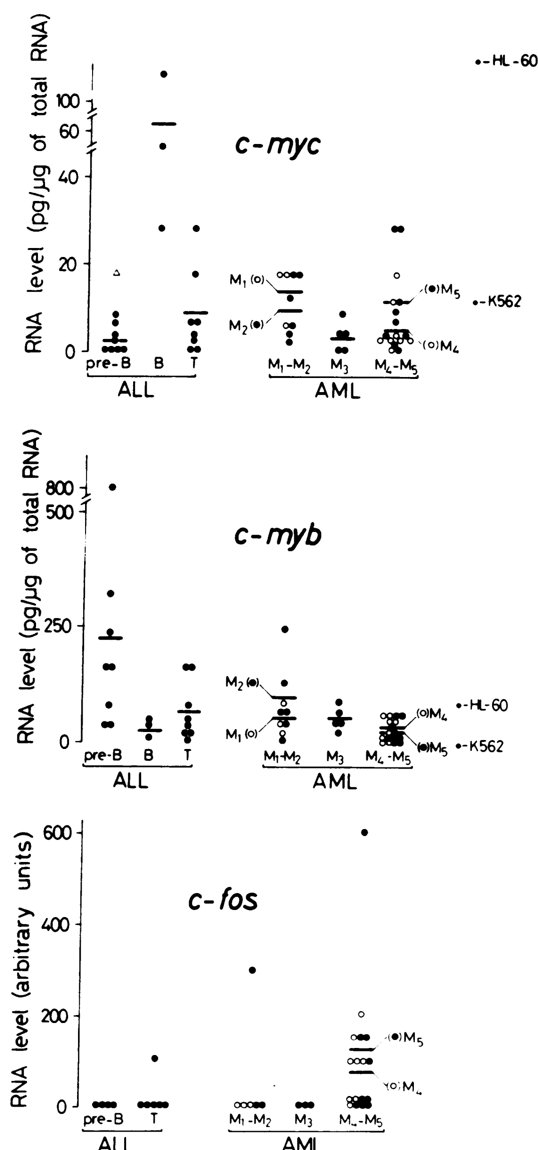

FIG. 2. Expression of c-*myc*, c-*myb*, and c-*fos* in ALL and AML. Subclassification of ALL and AML was by both surface antigen analysis and morphology (FAB) (see text). Horizontal bars represent mean values. Expression of c-*myc* and c-*myb* in HL-60 and K562 lines (derived from promyelocytic and chronic myelogenous leukemias, respectively) is indicated. Open triangle, level in the B-cell precursor ALL case (not included in pre-B-ALL mean value).

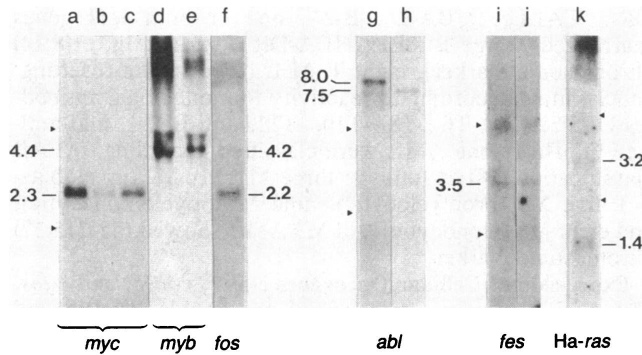

FIG. 3. Blot hybridization analysis of c-*myc*, c-*myb*, c-*fos*, c-*abl*, c-*fes*, and c-Ha-*ras* transcripts in total (lanes d, e, i, and j) or poly(A)$^+$ (lanes a, b, c, f, g, h, and k) RNA from acute leukemias. Lanes: a, HL-60 cells; b, pre-B-cell ALL; c, T-cell ALL; d, pre-B-cell ALL; e, T-cell ALL; f, M5 AML; g, K562 cells; h, pre-B-cell ALL; i, M3 AML; j, T-cell ALL; k, M4 AML. Transcript sizes (in kb) are indicated. Black triangles indicate positions of 28S and 18S rRNA.

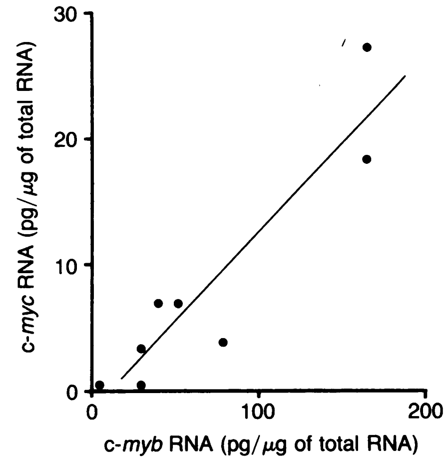

FIG. 4. Correlation ($r = 0.92$; $P < 0.001$) between expression of c-*myc* and c-*myb* in T-cell ALL.

pg. No correlation was observed within each group of ALL between levels of c-*myc* expression and either the rate of cell proliferation or the stage of differentiation (the latter as evaluated by both morphology and surface antigen pattern). The only exception was the very high expression in B ALL with 8;14 chromosome translocation, whereby c-*myc* transcription is deregulated due to gene rearrangement into the IgH locus (32).

Expression of c-*myc* in AML was variable as in ALL (Fig. 2), again without correlation with the FAB classification.

The c-*myb* gene was transcribed in ALL at variable levels, as a 4.4-kb mRNA, as assayed by blot hybridization; in some cases, a smear of possibly unspliced precursors was also detected (Fig. 3). The highest levels of expression were observed in pre-B ALL (233 ± 89 pg), which were 3- and 7-fold higher than in T ALL (69 ± 22 pg) and B ALL (34 ± 11 pg), respectively. In T-ALL cells, a strong direct correlation was observed between the expression of c-*myb* and c-*myc* (Fig. 4). Such correlation was not apparent in both pre-B and B-ALL groups.

Expression of c-*myb* in AML was variable. Although differences between subgroups were not significant by statistical analysis, the lowest values were apparently in M4/M5 (Fig. 2).

The c-*fos* gene was significantly transcribed only in M4 and M5 AML (Fig. 2). In both subclasses, ≈50% of samples showed significant expression of c-*fos* in RNA dots, with a clear correlation between level of c-*fos* transcripts and expression of monocytic-like surface antigen markers (data not shown). A 2.2-kb mRNA was detected in blots of electrophoretically fractionated poly(A)$^+$ RNA, together with a faint 4.2-kb band that may represent a precursor (Fig. 3).

Expression of c-*abl*, c-*fes*, c-*erbB*, and c-*src*. Expression of c-*abl* was low in ALL and in M1, M2, and M3 AML and was barely detectable in M4 and M5 AML, as compared to the level in the Philadelphia chromosome (Ph^1)-positive K562

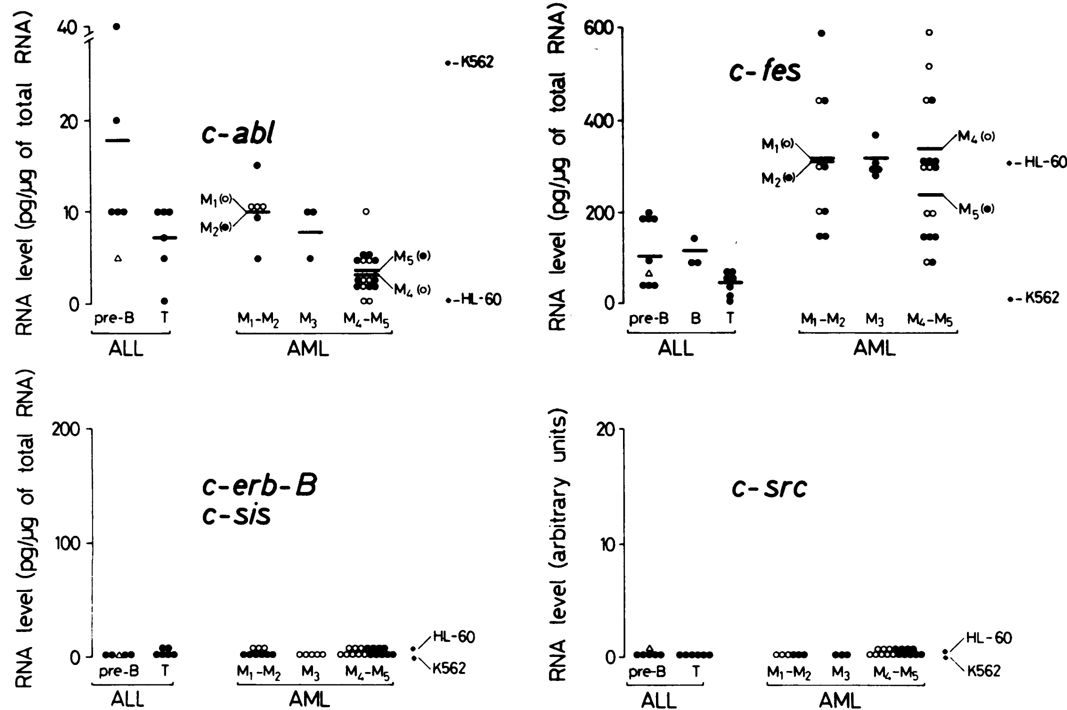

FIG. 5. Expression of c-*abl*, c-*fes*, c-*erbB*, c-*sis*, and c-*src* in ALL and AML (see legend to Fig. 2).

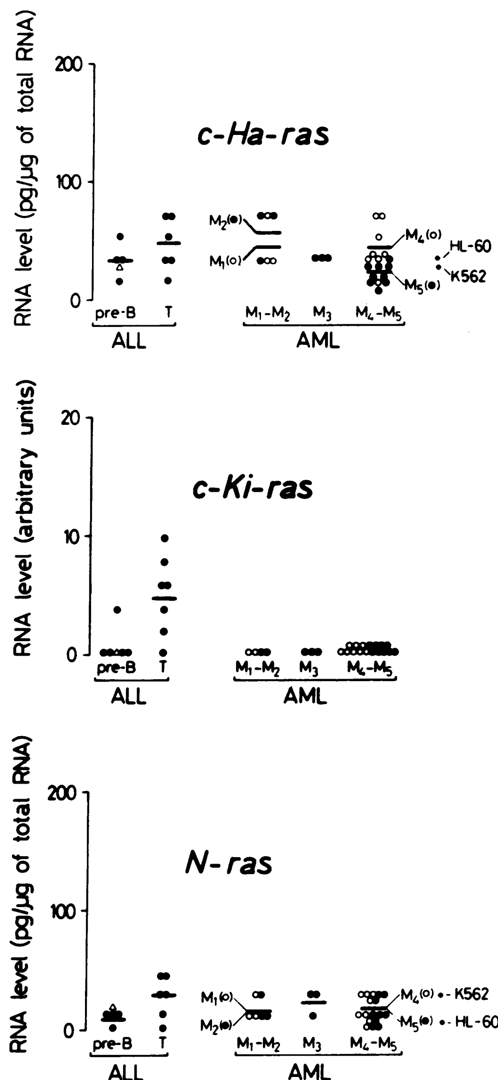

FIG. 6. Expression of c-Ha-*ras*, c-Ki-*ras*, and N-*ras* in ALL and AML (see legend to Fig. 2).

line (≈25 pg), in which the c-*abl* gene is amplified and overexpressed (36). A relatively strong signal was observed only in two cases of pre-B ALL (Fig. 5). However, one of them had a 9;22 chromosome translocation and may thus represent a B-lymphocytic progression of a previously unrecognized chronic myelogenous leukemia (CML). The size of the c-*abl* transcript was ≈7.5 kb. In K562 an abnormal band of ≈8 kb is predominantly expressed (Fig. 3) (36).

c-*fes* was expressed at highly variable levels as a 3.5-kb mRNA in virtually all subclasses of AML (Fig. 5): the average level was comparable to that in the promyelocytic HL-60 line (≈300 pg), which was in turn similar to the fairly homogeneous level in M3 AML. c-*fes* expression was also detected in ALL, as shown by analysis of both total RNA and electrophoretically fractionated poly(A)$^+$ RNA (Figs. 3 and 5), although at levels one-third to one-fourth those seen in AML.

Expression of c-*erbB* and c-*src* was undetectable in all samples.

Expression of c-*sis*. Transcripts of c-*sis* were never detected in any of the 51 AML or ALL samples.

Expression of c-Ha-*ras*, c-Ki-*ras*, and N-*ras*. Among *ras* genes, only c-Ha-*ras* was expressed at significant, although low, levels in both ALL and AML (Fig. 6). These levels are similar to those detected in non-neoplastic human cells, such as cultured fibroblasts, normal bone marrow cells, and lymphoblastoid lines (data not shown). The sizes of c-Ha-*ras* transcripts were 1.4 and 3.2 kb (Fig. 3). Expression of c-Ki-*ras* was detected only in T ALL (Fig. 6). The N-*ras* gene was transcribed at barely detectable levels in all leukemias.

Structure of Cellular Oncogenes in Acute Leukemia Cells. c-*myc*, c-*myb*, c-*fos*, c-*abl*, and c-*fes* were not amplified and showed a normal restriction pattern in all leukemic cells, as evaluated by Southern blot hybridization. The only detected structural abnormality was c-*myc* rearrangement in the three patients with t(8;14) B ALL (see ref. 32).

DISCUSSION

Evidence accumulated in recent years suggests a role for cellular oncogenes in establishment, maintenance, and progression of neoplastic growth, particularly for the hemolymphopoietic system (1, 4, 5, 10, 11). Amplification and/or enhanced expression of cellular oncogenes have been reported in a number of neoplastic hemopoietic lines (5). More significant, several cellular oncogenes are located at the breakpoints of chromosomal translocations associated with specific types of human leukemias or lymphomas [e.g., c-*abl* in the t(9;22) of Ph^1-positive CML; c-*myc* in t(8;14), t(8;22), and t(2;8) of Burkitt lymphoma or B ALL] (reviewed in ref. 37). However, a cause–effect relationship between cellular oncogene abnormality(ies) and oncogenesis in hemopoietic tumors has not been established (see ref. 38).

Previous studies on the expression of cellular oncogenes in lymphomas/leukemias have met with various limitations. A crucial aspect is that most observations have been carried out on neoplastic lines, which may not faithfully represent the original neoplastic clone(s) [e.g., amplification of c-*myc* in the promyelocytic line HL-60 and of c-*abl* in the CML-derived line K562 is normally absent in corresponding primary cells of M3 AML and CML, respectively (12)]. On the other hand, the limited number of studies carried out so far on primary cells are not easily interpreted, in view of the insufficient number of samples examined, the poor classification of neoplastic cells, and often the inadequacy of the molecular analysis (13–17).

We have therefore undertaken a systematic survey of the expression of 11 cellular oncogenes in a representative number of cases of ALL and AML, by hybridization analysis of RNA from purified, carefully classified neoplastic cells obtained prior to initiation of therapy.

Both c-*myc* and c-*myb* were expressed in all the leukemic cells. An intriguing observation is the class-specific pattern in ALL subtypes, particularly the inverse correlation between the quantitative expression of these oncogenes in pre-B, B, and T ALL. Conversely, their transcription shows a strong direct correlation within T-ALL samples.

Expression of the c-*fos* gene was strictly confined to AML of monocytic type. This finding is in line with observations obtained by chemical induction of leukemic lines, showing that c-*fos* expression is significantly linked to monocytic differentiation (39, 40).

Of those genes belonging to the "*src*-family" (i.e., those that encode membrane-associated protein kinases with putative or demonstrated growth factor-receptor function), only c-*fes* and c-*abl* were transcribed at detectable levels in leukemic cells. c-*fes* expression in AML is not surprising, in view of evidence suggesting that this gene might code for a protein related to the receptor of the granulocyte/macrophage colony-stimulating factor (41). However, c-*fes* was transcribed also in ALL, although at lower levels. This finding has not been reported in previous studies (42).

Protooncogenes coding for proteins not strictly related to hemopoietic differentiation (i.e., c-*sis* and c-*erbB*) are apparently not transcribed in leukemic cells.

The expression of Ha-*ras* and N-*ras* genes was very low in all samples, without significant differences from the levels in non-neoplastic human cells. It has been postulated that ras proteins exert their function as part of a receptor complex transducing mitogenic signals to the nucleus (43). Point mutations in critical sites may "activate" their oncogenic potential, even in absence of an altered rate of transcription (44). In particular, recent data indicate that the N-*ras* gene may be activated by mutation in AML (45). Our results suggest that the quantitative expression of this gene is not critically affected in leukemic cells.

An interesting finding is the T-ALL-specific expression of the Ki-*ras* gene. The significance of this phenomenon is still obscure: at present, we do not know whether it is a normal feature of proliferating T cells or a specific one of their neoplastic counterparts.

Our results provide a firm basis for future in-depth analysis of the role of cellular oncogenes in normal and neoplastic hemopoiesis. We suggest that the expression of cellular oncogenes in leukemic cells may reflect their proliferative activity (c-*myc*, c-*myb*), their differentiative state (c-*fos*), or the expression, at either normal or abnormal levels, of putative receptors for hemopoietic growth factors (c-*abl*, c-*fes*).

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