Expression of A-myb, But Not c-myb and B-myb, Is Restricted To Burkitt's Lymphoma, sIg$^+$ B-Acute Lymphoblastic Leukemia, and a Subset of Chronic Lymphocytic Leukemias

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The A-myb gene encodes a transcription factor that is related both functionally and structurally to the v-myb oncogene. Following our observations that A-myb is expressed in a restricted subset of normal mature human B lymphocytes, with the phenotype CD38$^+$, CD39$^-$, sIgM$^-$, we have now investigated the pattern of A-myb expression in neoplastic B cells representing the whole spectrum of B-cell differentiation and compared it to that of c-myb and B-myb. In a panel of 32 B-cell lines, A-myb was very strongly expressed in most Burkitt's lymphoma (BL) cell lines, but weak or negative in 2 pre-B acute lymphoblastic leukemia (ALL), 4 non-Hodgkin's lymphoma (NHL), 6 Epstein-Barr virus-immortalized lymphoblastoid cell lines, and 6 myeloma lines. Protein expression paralleled that of the RNA. We have also investigated A-myb expression in 49 fresh cases of B leukemias. Among 24 ALL, 6 were of the null and 11 of the common type and all these were negative for A-myb expression; on the other hand, all 7 B-ALL cases (sIg$^+$), as well as one fresh BL case with bone marrow infiltration, expressed A-myb. A-myb was undetectable in 4 prolymphocytic leukemias (PLL) but was strongly expressed in 5/20 (25%) of chronic lymphocytic leukemia (CLL) samples. In the latter A-myb did not correlate with phenotype or clinical stage. Finally, we have studied the progression of one case of CLL into Richter's syndrome and have found that the Richter's cells expressed about 25-fold less A-myb RNA than the CLL cells from the same patient. The pattern of c-myb and B-myb was clearly distinct from that of A-myb. C-myb and B-myb were expressed in all neoplastic groups, except in CLL cells. Thus, A-myb expression, unlike that of c-myb and B-myb, is restricted to a subset of B-cell neoplasias (in particular BL and sIg$^+$B-ALL) representative of a specific stage of B-cell differentiation. This expression may in part reflect expression of A-myb by the normal germinal center B cells that are the normal counterpart of these transformed B cells. The data presented strongly support a role for this transcription factor in B-cell differentiation and perhaps in B-cell transformation in some neoplasias.

THE A-myb gene belongs to the myb gene family.[1,2] This family includes the v-myb oncogene which is carried by the avian retroviruses AMV and E26 and which transforms cells of hematopoietic origin in vivo and in vitro in the chicken.[3-5] The cellular counterpart of v-myb is the c-myb proto-oncogene, which has been directly implicated in the regulation of hematopoietic proliferation and differentiation.[5-9] C-myb expression is mostly restricted to the hematopoietic system and correlates with both the immaturity and/or the proliferative state of these cells.[5,10] Both v-myb and c-myb are transcription factors.[11-13] They bind to a specific DNA sequence (PyAACG/TG) and activate transcription downstream of such sequences.[14] The transcriptional activity of v-myb is essential for and correlates with its oncogenicity.[5,15] More recently, two other members of the myb family, called A-myb and B-myb, have been cloned from human sources through their homology to c-myb.[1] Regions of homology include the DNA binding domain of c-myb and two other domains thought to be involved in the regulation of the transcriptional activity.[1,2] The A-myb protein has been shown to recognize the same DNA sequence as c-myb, but its transcriptional activity in vitro is 6- to 10-fold stronger than that of c-myb and comparable with that of the v-myb oncogene.[2,16]

Whereas B-myb is thought to be involved in the regulation of proliferation in many cell types,[5,17-19] little is known about the biologic function of A-myb. Its expression does not correlate with proliferation in mature leukocytes stimulated in vitro, unlike that of c-myb and B-myb.[18] A-myb was found to be expressed in a subset of human tonsillar B lymphocytes but was not detected at significant levels in most other peripheral mature leukocyte populations tested (monocytes, neutrophils, B lymphocytes) at rest or after in vitro stimulation.[18,20] Only resting T cells expressed A-myb relatively weakly, and this expression was lost upon mitogenic stimulation.[18] The phenotype of the tonsillar B cells expressing higher levels of A-myb was determined by analysis of fluorescence-activated cell sorter (FACS) sorted cells. A-myb expression could be shown to correlate with the phenotype CD38$^+$, CD39$^-$, or sIgM$^-$.[20] In addition, the CD38$^+$ subpopulation that expressed highest levels of A-myb was found to be highly proliferating, with over 95% of the cells in cycle (G1, S, G2/M).[20] Both the phenotype and cell-cycle status of the B cells expressing high levels of A-myb suggest that A-myb is induced during B-cell activation within germinal centers. This has been confirmed by in situ hybridization.[21] This is of particular interest in view of the fact that germinal center B lymphocytes undergo a series of crucial prolifera-

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tive and differentiative events: they proliferate at an extremely high rate, undergo Ig class switching, affinity maturation, differentiation to preplasma cells and apoptosis.[22] A-myb was recently found to be a strong transcriptional activator and is highly related in its structure and biochemical activity to c-myb and v-myb,[2,16] which are genes known to be involved in the control of hematopoietic proliferation/differentiation. Therefore, it is attractive to hypothesize that A-myb plays a role in one of the proliferative/differentiative processes taking place within germinal center B lymphocytes.

To determine whether the restricted A-myb expression during normal B-cell differentiation[20] is maintained also in neoplastic B cells, we set out to investigate what was the pattern of expression of the A-myb gene in a large panel of B-cell lines and fresh leukemic B-cell samples, which represent B cells blocked at different stages of differentiation, from pre-B cells to plasma cells. In addition, we have determined the pattern of expression of the c-myb and B-myb genes in the same samples. Although alterations of the c-myb gene have been reported in murine leukemias[23-25] and occasionally in human leukemias,[26-29] and its expression is thought to decrease during hematopoietic differentiation,[10,30,31] expression of c-myb has never been systematically investigated in neoplastic B cells. The pattern of B-myb is also completely unknown in these cells. The results show that A-myb is strongly expressed in the majority of Burkitt's lymphoma (BL) and sIg^+ B-acute lymphoblastic leukemia (B-ALL), as well as in approximately 25% of B-chronic lymphocytic leukemia (B-CLL) cells but not or very weakly in other neoplastic B cells studied. On the other hand, c-myb and B-myb were expressed in all mitotically active populations of neoplastic B cells, although in a heterogeneous manner within each group.

MATERIALS AND METHODS

Cell lines and cell cultures. The Chep and Eli BL lines and Epstein-Barr virus-immortalized lymphoblastoid cell line (EBV-LCL) were a kind gift of Dr A. Rickinson (CRC Laboratories, University of Birmingham, Birmingham, UK).[32] Several lines (Silti, DN-90, Cap, Capo) were established by A.R. Silti was derived from a BL patient and carries a t(2;8)(p11;q24) translocation. DN90 was established from a BL patient and carries a t(8;14)(q24;q32) translocation. Cap is a plasmablastic leukemia cell line. The OCI-LY cell lines were a kind gift of Dr Messner (Ontario Cancer Institute, University of Toronto, Canada).[33] OCI-LY3 has been described as a diffuse large cell lymphoma and OCI-LY 4,8 and 10 as immunoblastic lymphomas.[33] The other lines were obtained from the American Tissue Culture Collection (ATCC; Rockville, MD). All lines were grown in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Hyclone, Steril System, Logan, UT), glutamine (GIBCO, Paisley, Scotland), and 50 g/mL gentamicin (GIBCO).

The IM-9 line was stably transfected with the complete A-myb cDNA cloned in the pCEP4β vector (Invitrogen, Leek, Holland) by electroporation at 300 mV, 960 μFD (Gene Pulser; BioRad, Hercules, CA). Transfectants were selected in medium containing 350 μg/mL hygromycin (Boehringer, Mannheim, Germany).

Fresh leukemic samples. Bone marrow (BM), peripheral blood (PB), or lymph node samples were obtained with prior informed consent from patients with ALL, B-CLL, non-Hodgkin's lymphoma (NHL), or acute plasmablastic leukemias. The diagnoses were made according to standard procedures and French-American-British (FAB) classification. Richter's syndrome patient AV had been diagnosed to have CLL, Rai stage I in 1987. The white blood cell (WBC) count at diagnosis was 27×10^9/L and the phenotype of the CLL cells was λ^+, $CD5^+$. The patient did not require therapy until 1991, when combined chlorambucil/prednisone treatment was initiated. In May 1992, AV presented with a blastic progression typical of Richter's syndrome with massive lymphadenopathies and hepatomegaly. Biopsy of an axillary lymph node showed the presence of blastic lymphoid cells. RS was documented by the histologic analysis of the lymph node and immunophenotype of the blastic component.

RNA extraction and Northern blots. RNA was extracted by standard guanidium isothiocyanate and cesium chloride gradient purification.[18] Twenty micrograms of total RNA was run in 1% formaldehyde-agarose gels. The A-myb, c-myb, B-myb, and β-actin probes have been described previously[18] and were labeled with ^{32}P by standard nick-translation. The c-myb probe contained the third exon of human c-myb from *Cla* I to *Eco*RI (myc3RC).[34]

Western blots. The A-myb specific antiserum was raised in rabbits against a nonconserved fragment of the human A-myb protein produced in bacteria (a fragment from amino acid 218 to 482 showing 19% and 21% identity of amino-acids with c-myb and B-myb).[2] The antiserum used has already been described.[2,20] It was first purified by adsorption onto A-myb free bacterial lysates[2] and its specificity tested in Western blots containing either bacterially expressed A-myb or in fibroblasts transiently transfected with the A-myb complete or truncated cDNA or with the c-myb cDNA[2] (and unpublished data, November 1992). B cells, 2×10^6, were lysed in sodium dodecyl sulfate (SDS) loading buffer and run in a 10% SDS/polyacrylamide gel. The gels were electroblotted onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) for 8 hours at 35 V, according to standard procedures. The blots were incubated first in blocking solution (phosphate-buffered saline [PBS] containing 5% nonfat milk powder) overnight, then with the rabbit antiserum (1/1,000 in blocking buffer) for 1 hour and finally in diluted donkey-antirabbit conjugated to horseradish peroxidase (1/1,000; Amersham, Little Chalfont, Buckinghamshire, UK). Between each incubation the blots were washed three times for 10 minutes in PBS containing 0.5% NP40. Detection was performed using the ECL chemiluminescence system (Amersham).

Densitometry of Northern blots. The A-myb hybridization signals from selected blots were scanned with a GS300 Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA) using the GS370, version 2.0 program run on a MacIntosh Plus computer (Apple Computer, Inc, Cupertino, CA). The ethidium bromide–stained 28S RNA content of each lane was quantified by densitometric analysis on an image analyzer (IBAS 2.0 software) (Kontron/Zeiss, Oberkochen, Germany) running under MS-DOS environment (PC 80286 processor). The signals from different blots were normalized for (28S) RNA content and reference RNA (ie, an identical positive RNA, usually RNA from buoyant tonsillar B cells or a BL line, present in the different blots).

Indirect immunofluorescence and cell sorting. The CD3, CD20, CD5, and anti-κ and λ antibodies were obtained from Becton Dickinson (Mountain View, CA). The HD 50 (CD23) antibody was a kind gift of Dr G. Moldenhauer (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Monoclonal antibody (MoAb) AC2 (CD39) was a kind gift of Dr A. Rowe (CRC laboratories, University of Birmingham, Birmingham, UK). Antibody 38.13 (CD77) was kindly sent by Prof T. Tursz (Institut Gustave-Roussy, Villejuif, France). MoAbs BerH2 (CD30) and Ki-24 (CD70) were a generous gift of Dr H. Stein (Institute of Pathology, University of Berlin, Berlin, Germany). Cells were stained by standard indirect immunofluorescence procedures using a fluorescein-labeled goat-antimouse anti-

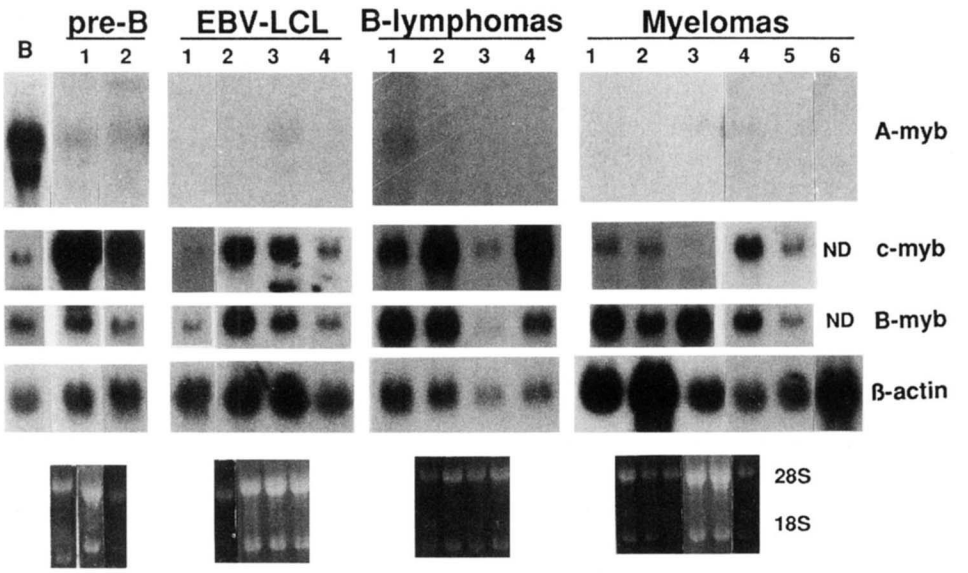

Fig 1. Myb mRNA expression in human B-cell lines. RNA was extracted from a panel of B-cell lines representing different stages of B-cell differentiation. Lane B, control RNA from normal tonsillar buoyant B cells; pre-B ALL: 697 (1), Nalm 6 (2); EBV-LCL: PCK3 (1), CESS (2), Laz156 (3), Capo (4); B lymphomas: OCI-LY3 (1), OCI-LY4 (2), OCI-LY8 (3), OCI-LY10 (4); myelomas: Hand (1), CBI.3 (2), Grafstein (3), RPMI 8226 (4), IM-9 (5), and Cap (6). The same blots were hybridized successively with the indicated ^{32}P-labeled probes. The photographs of the ethidium stained blots are also shown. ND, not done.

body (Technogenetics, Milan, Italy). Analysis and cell sorting was performed on a FACS (FACS Star Plus, Becton Dickinson, Sunnyvale, CA) as described.[20]

Semi-quantitative polymerase chain reaction (PCR) analysis. Total RNA from cell populations sorted on the FACS was purified on a standard isothiocyanate/cesium chloride gradient. Six or 25 ng of total RNA were reverse transcribed (RT) in 20 μL. Three microliters of RT product was amplified in duplicate for 20 and 25 cycles with β-actin and A-myb specific primers, respectively, as described.[20] Ten microliters of amplification product was run on a 1% agarose gel, blotted onto nitrocellulose and hybridized with ^{32}P-labeled β-actin and A-myb specific probes, respectively. The data were quantified on the GS300 scanning densitometer (Hoefer Scientific Instruments).

RESULTS

Expression of A-myb in a panel of B-cell lines. In a previous study, we had found that normal highly proliferating tonsillar B lymphocytes with the phenotype CD38$^+$, CD39$^-$, sIgM$^-$ express high levels of A-myb.[20] On the other hand A-myb, unlike c-myb and B-myb, was not induced during proliferation of tonsillar B cells in vitro induced by several polyclonal B-cell activators, suggesting that expression of A-myb was specific for a particular differentiation stage of B cells or for in vivo– but not in vitro–induced proliferation.[18,20] Therefore, we set out to investigate the pattern of A-myb expression in neoplastic B cells. We tested A-myb RNA expression in a panel of B-cell lines, including 2 pre-B lines, 6 EBV-LCL, 4 NHL, 6 myelomas, and 14 BL lines representing the whole spectrum of B-cell differentiation. A-myb was very weak or negative in all 18 non-BL lines, but was very highly expressed in two thirds of the BL cell lines (Figs 1 and 2): 9 of 14 BL lines (64%) showed very high A-myb expression, 1 (7%) (DN90; lane 12) expressed intermediate levels of A-myb, and 4 were weak or negative (29%) (ST486, Pollik, Namalwa, JD38, lanes 3, 6, 10, and 11, respectively, Fig 2). In several blots, RNA from normal buoyant tonsillar B cells was used as a positive control (lanes B of Figs 1 and 2). The pattern of A-myb expression in different cell lines was stable over an extended time of culture and thus did not reflect a particular state of the cells or the growth conditions, but rather was characteristic of the cell lines (data not shown). To further demonstrate the specific expression of A-myb in BL rather than EBV-LCL, we have also tested pairs of BL and EBV-LCL, both EBV$^+$ and originating from the same patients (Eli and Chep).[32,35] As shown in Fig 2, A-myb is specifically and strongly expressed in the BL but not EBV-LCL lines from both of these patients.

Of note is that in all cases, the size of the major A-myb transcript (about 5 kb) appeared identical to that observed in normal tonsillar B lymphocytes run in parallel. In all cases, a weaker band of about 10 kb is observed (Fig 2). Thus, there is no evidence of gross structural abnormality of A-myb in these human neoplasms as detected by Northern analysis.

Both Figs 1 and 2 show the results of hybridization with the β-actin probe and the photographs of the ethidium bromide–stained gels demonstrate that all lanes contained comparable amounts of RNA. To accurately quantify A-myb expression, we selected those blots that contained an identical RNA sample (RNA from buoyant tonsillar B cells, ie, a reference RNA) that could be used to compare the hybridization signals from different blots with each other. Quantification of 25 of the 32 cell lines could thus be performed by densitometric analysis. The A-myb signals were normalized for the quantity of total RNA, as determined by densitometric analysis of the 28S RNA, and the data are shown in Fig 3. The data clearly show that A-myb expression in most BL lines is 10- to 100-fold stronger than in other cell lines, although it shows some variability between cell lines. A-myb expression in all the other lines is low or undetectable.

C-myb and B-myb share some functional and structural homologies with A-myb. Therefore, we hybridized the same blots with the c-myb and B-myb probes. Most cell lines of all neoplastic groups were positive for both c-myb and B-

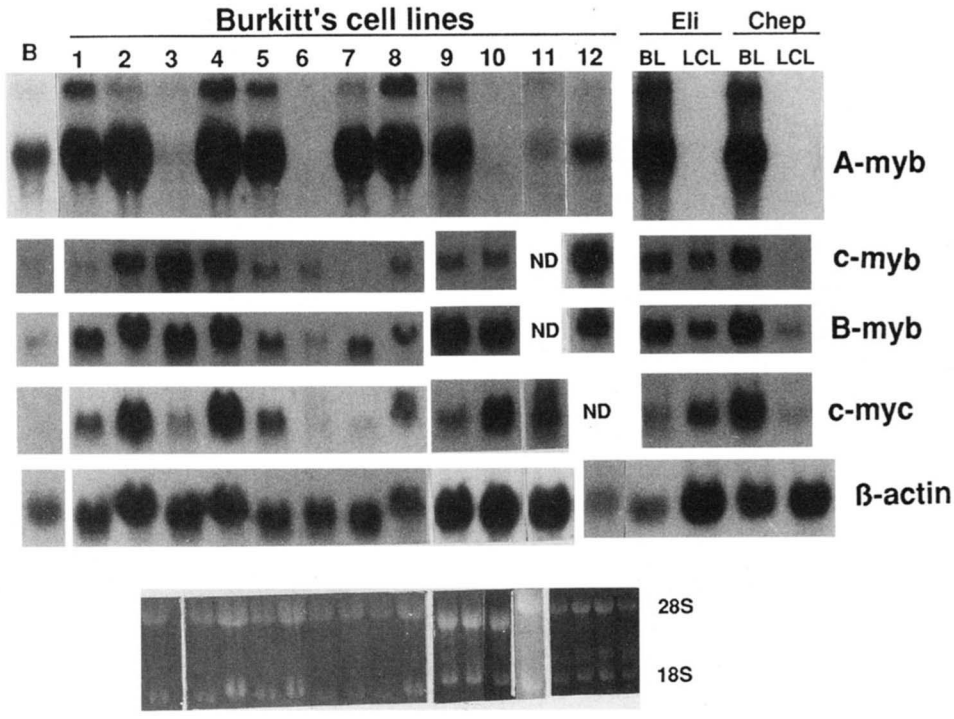

Fig 2. Myb and c-myc mRNA expression in BL lines. Northern analysis of total RNA extracted from the following BL lines: Ramos (1), MC116 (2), ST486 (3), BL2 (4), Daudi (5), Pollik (6), Silti (7), P3HR1 (8), Raji (9), Namalwa (10), JD38 (11), DN90 (12). BL and EBV-LCL from patients Eli and Chep. Lane B, control RNA from normal tonsillar buoyant B cells. The blots were hybridized in succession with the indicated probes. The photographs of the ethidium-stained blots are also shown. ND, not done.

myb, although with some heterogeneity within each group (Figs 1 and 2). Thus, A-myb expression was clearly different and more restricted than that of c-myb and B-myb.

Since the A-myb gene has been localized on chromosome 8q22-23, that is relatively close to c-myc (8q24),[36] we have performed Southern blot analysis using the A-myb complete cDNA as probe after standard or pulse-field gel electrophoresis of DNA from several BL cell lines, including cell lines that rearrange far upstream of c-myc (Daudi, AG876),[37,38] but could not detect any rearrangement of A-myb (data not shown). We also hybridized the BL samples with a c-myc probe to determine whether A-myb levels correlate with those of c-myc. The results shown in Fig 2 demonstrate that there is no direct correlation between A-myb and c-myc expression in the cell lines. For example, one line negative for A-myb (line 10) has a rather strong c-myc signal, and others that show strong A-myb expression (lines 7, 9, Eli) have relatively weak c-myc.

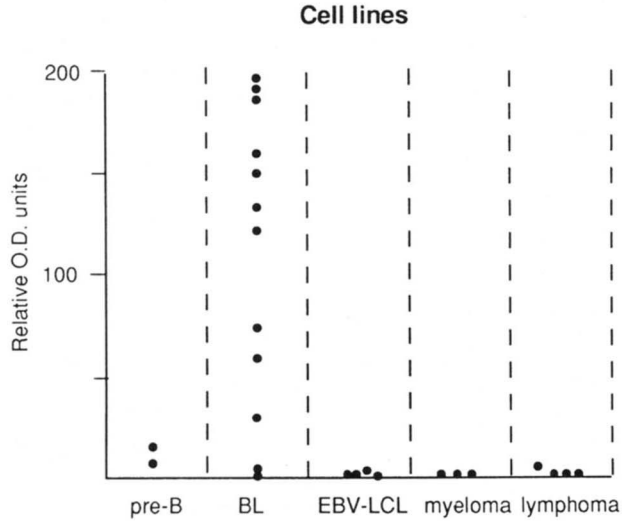

Fig 3. Densitometric analysis of A-myb expression in B-cell lines. The A-myb signals from several blots containing a reference RNA were measured by densitometric analysis and normalized for both total RNA content and against a reference RNA. The results are presented as arbitrary optical density (O.D.) units.

Expression of the protein correlates with that of the RNA. To determine whether the levels of A-myb mRNA correlate with those of its protein, a number of cell lines were examined for A-myb protein expression in Western blots with our purified rabbit antiserum. Although this antiserum had been previously characterized and shown to specifically recognize the A-myb protein,[2,20] its specificity was further verified in the myeloma cell line IM9 stably transfected with the complete A-myb cDNA. As shown in Fig 4A and as observed previously, the A-myb protein runs as a doublet of about 90 kD.[2,39] By analogy with c-myb, the doublet probably represents different phosphorylation forms of A-myb,[40] the relative amounts of which can vary in different cells (Fig 4A and J.G., M.L., unpublished observations, April 1993-March 1994). C-myb and B-myb have a different molecular weight (of 75 and 93 to 100 kD, respectively)[41,42] and are not recognized by the antiserum that was raised against a nonconserved region of the A-myb protein.[2] We next tested A-myb protein expression in selected cell lines: these were 4 BL A-myb positive in Northern analysis, 3 BL negative for A-myb RNA expression, 3 EBV-LCL, and 2 myelomas. As shown

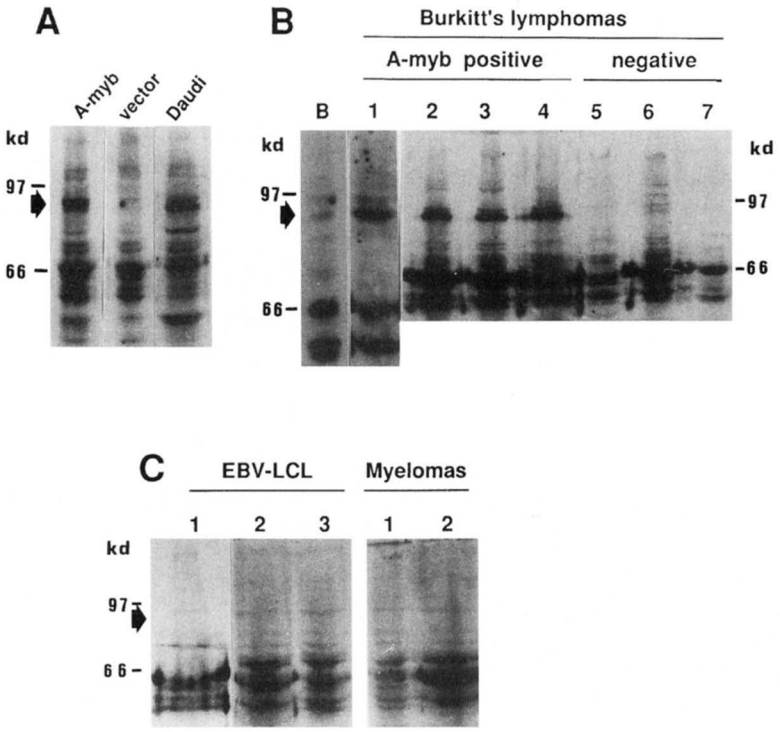

Fig 4. A-myb protein expression in B-cell lines. (A) The specificity of the polyclonal anti-A-myb antiserum was tested using cellular extracts from the IM-9 myeloma cell line stably transfected with the complete A-myb cDNA or with the empty vector. An extract from the Daudi cell line was run in parallel. The position of the molecular-weight markers are shown and that of the 90-kD A-myb doublet is indicated with a black arrow. (B and C) Western analysis of cellular extracts from normal CD38$^+$ tonsillar B cells (lane B) and from different cell lines. BL lines: Daudi (1), Ramos (2), Raji (3), Silti (4), Pollik (5), Namalwa (6), ST486 (7). EBV-LCL: CESS (1), Capo (2) Eli (3). Myelomas: IM-9 (1), RPMI 8226 (2). The position of the A-myb specific band is indicated by an arrow.

in Fig 2B and C, A-myb protein expression paralleled that of its RNA in all 12 cell lines tested. In all cases the protein was nuclear as expected[2] (data not shown). Thus, both the protein and the RNA of A-myb are highly expressed in most BL lines but not in the other B-cell lines tested. It is worth noting that, although the size of the protein was identical to that of normal B cells, the levels of expression were 5 to 10 times higher in BL lines relative to that observed in purified CD38$^+$ normal B lymphocytes (Fig 4B, lane B). Because our antiserum does not recognize the native protein in immunocytochemistry, it is not possible at present to assess the proportion of the cells in either BL lines or CD38$^+$ tonsillar B cells expressing the A-myb protein. Therefore, we cannot determine whether A-myb is overexpressed at the single cell level.

The pattern of A-myb expression in BL cell lines does not correlate with the presence of EBV, the type of chromosomal translocation, or the phenotype of the cells. Because BL shows heterogeneity at several levels, in particular in its endemic or sporadic forms, with respect to the type of translocation [t(8;14), t(2;8), t(8;22)], presence or absence of EBV,[43] pattern of growth (as clumps or single cells),[31] and phenotype,[32,43-45] we chose a series of 6 BL lines, 3 of which (Daudi, Raji, Silti) show high and the other 3 (Namalwa, ST486, Pollik) show low/negative A-myb expression and compared A-myb expression with the known and observed characteristics of the lines. As shown in Table 1, A-myb expression did not correlate with the form of the original disease (endemic or sporadic), type of translocation [t(8;14) *v* t(2;8) or t(8;22)], presence of EBV, or pattern of growth. It did not appear to correlate with the presence or absence of particular markers known to vary among different BL lines (CD10, 23, 30, 39, 70, 77)[43-45] or classification within the previously defined phenotypic groups.[32]

Because c-myc rearrangement and consequent deregulation is thought to be the major factor in the pathogenesis of BL,[43] we wondered whether introduction of a deregulated c-myc gene in EBV-LCL could lead to increased expression of A-myb more similar to that of BL cell lines. Therefore, we tested in Northern analysis a series of EBV-LCL clones that had been stably transfected with a construct carrying the c-myc gene exons 2 and 3 downstream of the SV40 promoter.[34,46] The cell lines used had been shown previously to express intermediate to high levels of the exogenous c-myc protein.[34,46] However, in none of the transfected lines could we detect A-myb expression (data not shown), suggesting that the deregulated expression of c-myc is not sufficient to induce A-myb expression in this cellular context.

Analysis of A-myb expression in fresh leukemic samples. To further extend our analysis of A-myb in neoplastic B cells, we also examined a large panel of fresh B-cell leukemias. Non-T lineage ALL can be classified according to their phenotype in 3 subtypes, null (CD19$^+$, CD10$^-$, sIg$^-$), common (CD19$^+$, CD10$^+$, sIg$^-$), and B (CD19$^+$, CD10$^+$, sIg$^+$).[47] Cells of the sIg$^+$ B-cell subtype invariably have a morphology and phenotype indistinguishable from those of BL cells and have been shown to carry c-myc translocations characteristic of BL cells.[47] Thus, we screened 24 ALL samples, of which 6 were null, 11 were common, and 7 were of the sIg$^+$ B-cell subtype. As shown in Fig 5, all common and null ALL were very weak or negative for A-myb expression, whereas all 7 B-ALL samples were clearly positive for A-myb mRNA (Fig 6, lanes 1 through 7). Of note is that the normal BM control sample was negative (Fig 6). In addition

Table 1. Characteristics of A-myb Positive and Negative BL Lines

					Surface Phenotype							
Line	Type	Translocation	EBV	Growth	CD10	CD23	CD30	CD39	CD70	CD77	Group*	A-myb
Daudi	E	8;14	+	SC	+++	−	−	−	+	+	I	High
Raji	E	8;14	+	SMC	+++	++	+	−	++	+	II	High
Silti	S	2;8	−	LC	+++	−	+	−	+	++	I/II	High
Namalwa	E	8;14	+	SC	+++	−	−	−	++	−	I/II	Barely detectable
ST486	S	8;14	−	SC	+++	−	−	−	+	+	I/II	Barely detectable
Pollik	S	8;14	+	LC	−	−	++	+++	++	−	III	Negative

Abbreviations: E, endemic; S, sporadic; SC, mostly single cells; SMC, small to medium clumps; LC, large clumps; −, <5% positive cells; +, 6%-20%; ++, 21%-80%; +++, >80% positive cells.

• Phenotypic group.[26]

the BM sample collected during the leukemic phase of a BL patient was also strongly positive for A-myb (Fig 6, lane 8). In all blots, a positive control consisting of RNA from a BL cell line was included to control for the level of hybridization. We conclude that also among fresh leukemic samples, A-myb expression is restricted to cells of the more mature B (sIg^+) type. It is of particular interest that all cases of B-ALL tested so far were found to be positive for A-myb expression, suggesting that this may be a constant characteristic of this subtype of B-cell leukemias.

The same blots were hybridized with the c-myb and B-myb probes. C-myb and B-myb were well expressed in most samples, regardless of ALL subgroup. As for the cell lines, some variability of expression was observed within each subgroup (Figs 5 and 6). Thus, also in fresh cases of ALL, A-myb expression is restricted to a particular subset whereas that of c-myb and B-myb is not.

To extend the analysis of A-myb expression to other mature B-cell leukemias that could represent different B-cell differentiation stages and/or subsets, we tested 20 B-CLL and 4 PLL samples for A-myb RNA expression. As shown in Fig 7, A-myb expression was heterogeneous in the B-CLL samples: out of 20 cases, 5 showed strong expression (25%, cases 1, 9, 16, 17, and 19), 5 were weakly positive (25%, cases 4, 7, 10, 13, and 15) and all the others (50%) were negative for A-myb. The expression of A-myb in CLL correlated neither with Rai stage nor with other parameters such as blood cell counts (Table 2) or the CD38, CD39 phenotype of the CLL cells (data not shown). In addition, A-myb expression seemed relatively stable over time in that two patients who had shown intermediate or negative A-myb expression were found to maintain these characteristics several years following the first sampling and analysis (data not shown). A-myb expression in 13 ALL and 12 CLL samples could be quantified by densitometric analysis because they were present on blots that carried an identical positive RNA sample to use as reference RNA to compare the different blots. The results are shown in Fig 8 and clearly demonstrate that A-myb is expressed at much higher levels in the B-ALL relative to the null and common ALL samples and at comparably high levels also in a small subset of CLL.

C-myb and B-myb were also tested on the same blots.

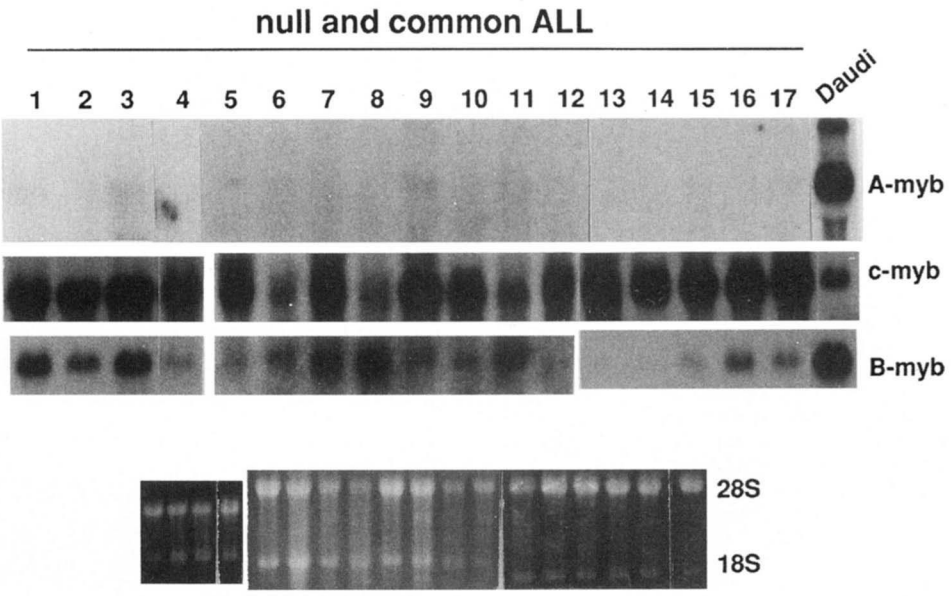

Fig 5. Myb mRNA expression in null and common ALL. Northern blot analysis of total RNA extracted from fresh BM samples of ALL patients. A representative positive control is shown on the right (Daudi cell line RNA). Lanes 1 through 5 and 9 contained RNA from null ALL samples and lanes 6 through 8 and 10 through 17 from common ALL. The blots were hybridized with the indicated probes. The photographs of the ethidium bromide–stained blots are also shown.

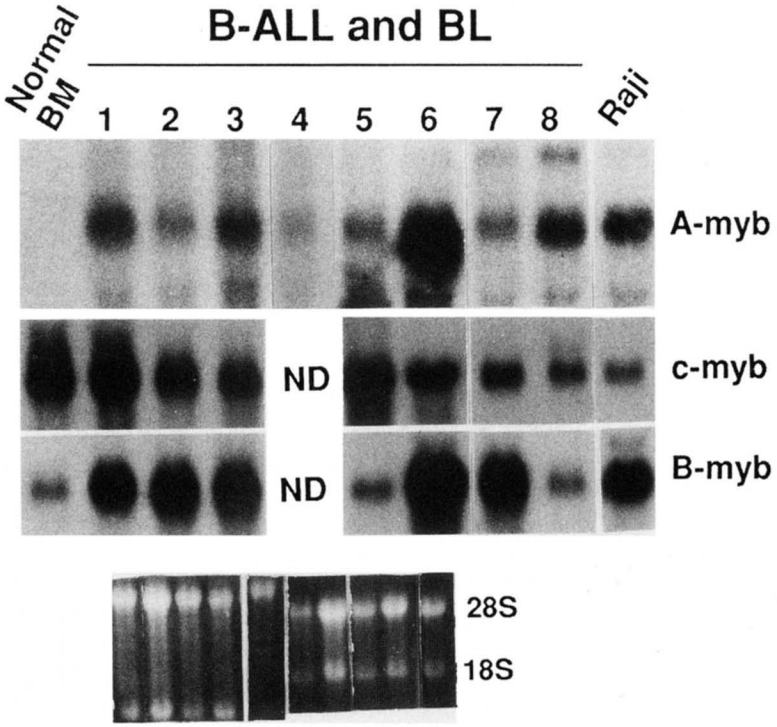

Fig 6. Myb mRNA expression in fresh B-ALL and BL. RNA from 7 slg$^+$ B-ALL (lanes 1 through 7) and from 1 BL patient with BM involvement (lane 8) were analyzed in Northern blots. RNA from a normal BM sample (BM) was also used as a control. The blots were hybridized with the indicated probes. The photographs of the ethidium bromide–stained blots are also shown.

The results show that c-myb was weakly expressed in two patients (cases 7 and 15) and was strong only in one case (number 13) (Fig 7). C-myb expression did not correlate with A-myb. B-myb, on the other hand, was undetectable in all 20 cases. Thus, again in the case of CLL, A-myb expression was distinct from that of the other two myb genes.

Nine of the 20 CLL samples were also hybridized with the c-myc probe; C-myc expression in CLL was variable but did not correlate with A-myb or c-myb expression (data not shown). Finally, 4 PLL samples were collected and tested for A-myb expression. All cases were negative (Fig 9).

Loss of A-myb expression during progression of a CLL case in Richter's syndrome (RS). Our present and previous data show that A-myb expression correlates with a restricted stage of B-cell differentiation and not simply with proliferation. In 3% to 5% of patients with the CLL, this neoplasm progresses into a more malignant large cell lymphoma called RS.[48] The large Richter's cells have altered morphology and in part phenotype relative to the CLL cells, but they are generally derived from the same clone.[49-54] Therefore, we wondered whether the clonal evolution observed in RS could also be associated with a change in A-myb expression. One

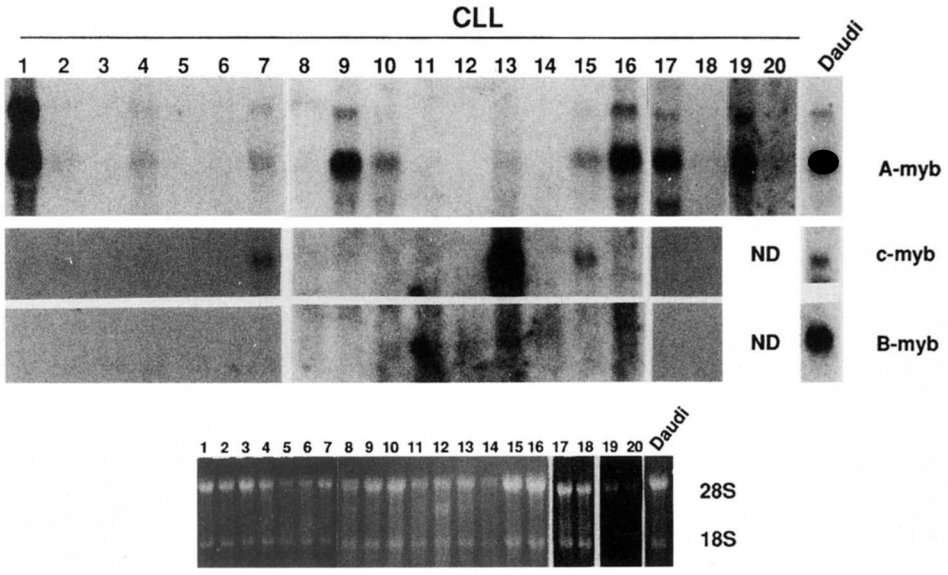

Fig 7. Myb mRNA expression B-CLL. Total RNA was extracted from blood samples from CLL patients and analyzed for A-myb expression in Northern blots. The blots were hybridized with the indicated probes. The photographs of the ethidium bromide–stained blots are also shown.

Table 2. Characteristics of B-CLL Patients

Patient No.*	RAI Stage	k/λ	CD5	Leukocytes/μL	A-myb mRNA†
17	I	κ	+	32,000	++
1	II	κ	+	240,000	+++
12	II	λ	+	61,000	−
16	II	κ	−	57,000	++
2	III	κ	+	68,000	−
3	III	λ	−	24,000	−
8	III	κ	+	280,000	−
9	III	ND	+	164,000	++
14	III	ND	+	23,000	−
19	III	κ	+	82,000	++
20	III	λ	+	148,000	−
10	IV	κ	+	129,000	+
18	IV	κ	+	100,000	−

* The patient number corresponds to those of Fig 4.

† The +/− signs refer to the intensity of the A-myb signal as shown in Fig 4.

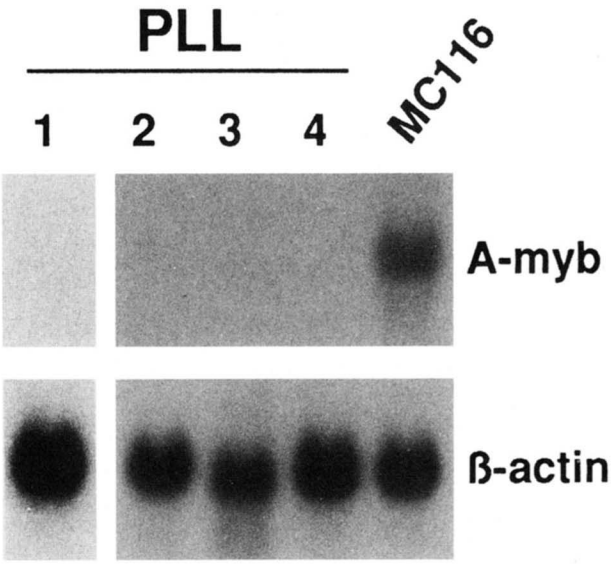

Fig 9. A-myb mRNA expression in B-PLL. Four B-PLL samples were analyzed for A-myb expression by Northern analysis. The hybridization results with A-myb and the β-actin probe as control are shown.

case of RS presented with both large and small lymphocytes in the PB clearly separable by FACS analysis on the basis of size. As shown in Table 3, phenotypic analysis of these two populations showed that the small cell population contains about 40% T lymphocytes ($CD3^+$) and 56% $CD20^+$, $CD5^+$, λ^+ CLL B cells. On the other hand, the large, blast cells were essentially all $CD20^+$, λ^+, and $CD5^-$ Richter's B cells. The light-chain isotype suggested that the Richter's cells were a clonal evolution of the CLL, as reported for the majority of Richter's cases.[49-54] To determine whether evolution of the CLL was accompanied by a change in A-myb expression, we separated the small and large $CD20^+$ B cells by cell sorting on the FACS and purified total RNA from these cells. The levels of A-myb expression were measured by a quantitative PCR method used successfully previously.[20] As a standard the actin gene was amplified from the same reverse transcribed samples. As shown in Fig 10, although equivalent levels of actin could be seen in both small and large B cells, the levels of A-myb were approximately 25-fold lower in the large B (Richter) B cells relative to the small (CLL) B-cell population. These data show that the evolution into RS in this patient was accompanied by a drastic reduction in A-myb gene expression.

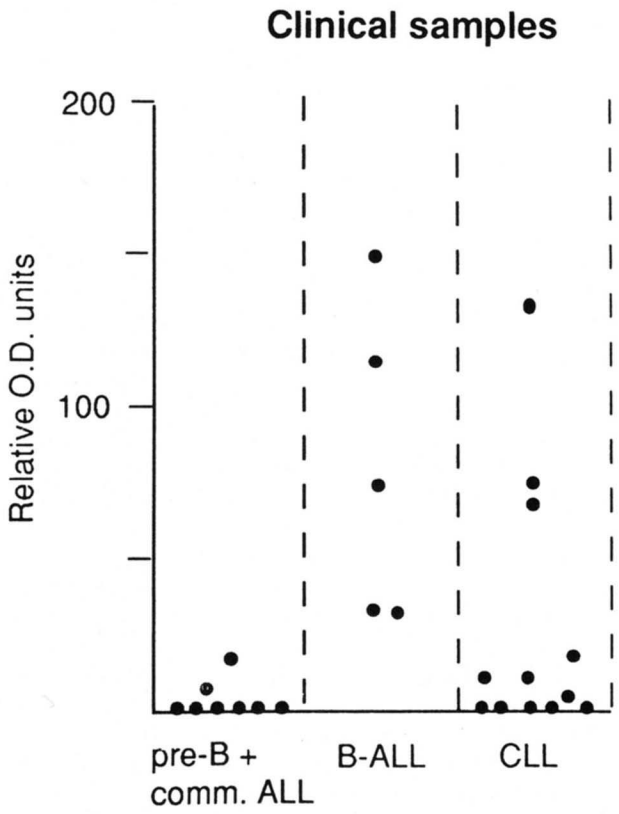

Fig 8. Densitometric analysis of A-myb expression in ALL and CLL. The A-myb signals from several blots containing a reference RNA were measured by densitometric analysis and normalized for both total RNA content and reference RNA. The results are presented as arbitrary O.D. units.

Table 3. Phenotype of Small (CLL) and Large (Richter) Mononuclear Cells of RS Patient

	% Positive Cells	
Surface Antigen	Small	Large
CD20	56.4	93.3
CD19	52.4	91.8
CD3	42.0	1.4
CD14	1.0	4.0
κ	15.2	11.6
λ	58.7	95.7
CD10	<1	<1
CD5	77.3	4.7

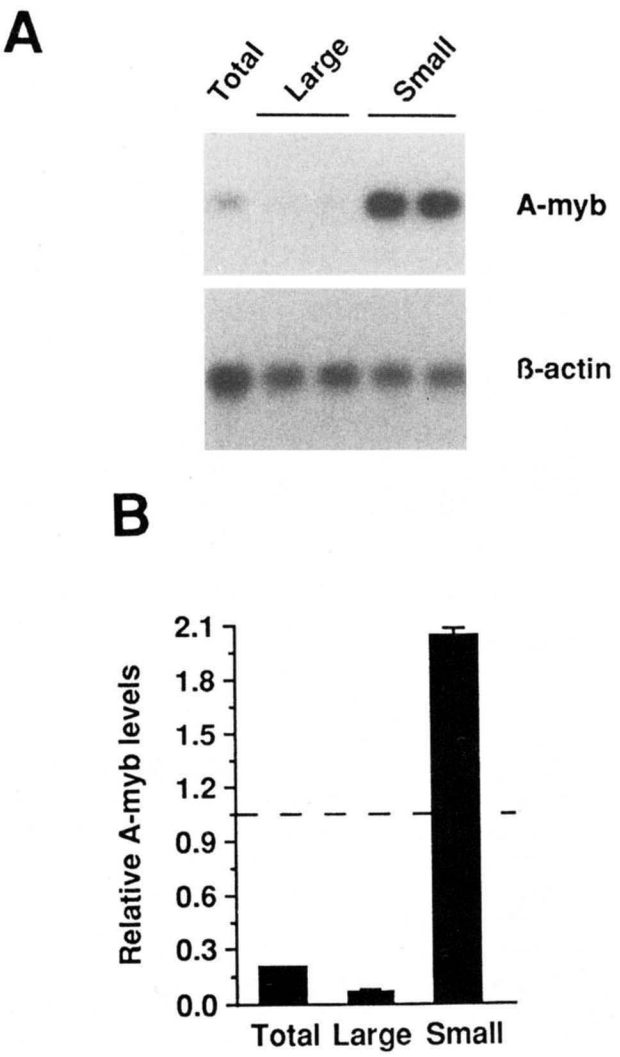

Fig 10. Expression of A-myb in the CLL and Richter's cells from the same patient. PB mononuclear cells from one RS patient were stained with an anti-CD20 antibody. The small and large CD20$^+$ populations were isolated by sorting on the FACS and expression of A-myb or β-actin was analyzed by semi-quantitative PCR amplification of 6 ng of purified total RNA from each sample in duplicate. RNA from the unsorted cells was also amplified in parallel (Total). The amplification products were run on an agarose gel, blotted, and analyzed by hybridization with A-myb or β-actin specific probes. The results of the hybridization are shown in (A). The β-actin and A-myb specific signals for each population were quantified by densitometry and the results are shown in (B).

DISCUSSION

In this report, we have described the pattern of A-myb gene expression in an extended panel of human B leukemia and lymphoma cell lines and fresh samples, representing different stages of B-cell differentiation and/or B-cell subsets. A-myb expression was also compared with that of the related c-myb and B-myb genes in the same samples. The results show that A-myb expression, unlike that of c-myb and B-myb, is restricted to cells with a Burkitt-like morphology, that is to BL and ALL of the B (sIg$^+$) type, but is absent from most other leukemias. Seventy-one percent of BL lines expressed intermediate to very high levels of A-myb, either as RNA or as protein, and 7 of 7 B-ALL (sIg$^+$) as well as one fresh BL sample tested were also clearly positive for A-myb. Other fresh leukemia samples tested, including 17 null and common ALL, 4 PLL, or B-cell lines including 6 myeloma, 4 NHL, and 2 pre-B ALL did not express A-myb significantly. Perhaps surprisingly, 25% of the cases of CLL also showed strong A-myb expression. Finally we have shown that the evolution of a CLL case into RS was accompanied by a 25-fold decrease in A-myb expression.

A-myb expression in BL cells was very strong in most cell lines but weak or negative in some of them (29%). This heterogeneity did not correlate with any of the parameters considered (type of translocation, presence of EBV, phenotype, growth pattern). A lack of A-myb expression could have been due to loss of expression during culture in vitro because it is well known that many phenotypic and genetic alterations take place during long-term in vitro culture.[32,55] Our finding that all 7 sIg$^+$ B-ALL cases as well as 1 fresh BL sample with BM involvement were positive for A-myb expression suggests that the expression of this gene may be a constant characteristic of these types of leukemias/lymphomas.[47] A more accurate evaluation of whether A-myb expression is always associated with BLs/B-ALL will require analysis of a larger number of cases of these relatively rare neoplasias.

Our finding that A-myb expression is restricted nearly exclusively to BL and mature B-ALL cells is particularly interesting because the neoplastic B cells from these two diseases are phenotypically and morphologically identical and both present the typical translocations involving the c-myc and Ig genes.[43,47] Our previous data had shown that in normal tonsillar B lymphocytes, A-myb is strongly expressed in activated germinal center B lymphocytes with the phenotype CD38$^+$, CD39$^-$, sIgM$^-$.[20] Also, the phenotype of BL and B-ALL cells, in particular with respect to expression of CD38, CD39, CD77, CD10, suggests that they derive from germinal center (GC) cells.[44,45,47,56] In addition, both BL and germinal center B cells are prone to apoptosis, again suggesting that BL derive from GC B cells.[57,58] Our data on A-myb expression add further evidence to this hypothesis and suggest that A-myb expression in BL and B-ALL may at least in part reflect the phenotype of their normal B-cell counterpart. The study of the expression of A-myb may thus help understand the biological characteristics of the normal and neoplastic B cells that express this transcription factor as well as the pathogenesis of BL and B-ALL.

A-myb RNA expression was high in BL and B-ALL. Also at the protein level, A-myb expression was 5- to 10-fold higher in BL lines than in purified CD38$^+$ B cells. These data suggest that A-myb may be overexpressed in BL and B-ALL cells relative to normal B cells. However, the demonstration of such hypothesis will require analysis of A-myb expression at the single-cell level using immunocytochemistry and/or in situ hybridization. Such studies are in progress. In this respect it is rather intriguing that the A-myb genome has been localized on chromosome 8q22-23,[36] which is rela-

tively close to the c-myc gene (8q24).[43] However, standard or pulse-field Southern analysis using the A-myb complete cDNA as probe has not shown any rearrangement of A-myb and has not allowed to evaluate the real distance between A-myb and the breakpoints located far upstream of c-myc present in some cell lines (Daudi, AG876).[37,38] Also, deregulation of c-myc as tested in EBV-LCL transfected with a c-myc construct did not lead to induction of A-myb expression, suggesting that a deregulated c-myc is not directly causative of A-myb expression, at least in this cellular background.[34,46] We have also investigated in the cases of BL lines and CLL whether A-myb expression correlated with that of c-myc, but found no evidence that this is the case. Thus, we have no direct evidence of a relationship between c-myc rearrangement or regulation and A-myb expression.

Our finding that A-myb was also strongly expressed in about 25% of CLL samples was rather surprising because these cells have a very different phenotype, behavior and cellular origin from BL or B-ALL cells.[48,59] Indeed, it has been hypothesized that CLL cells originate from a distinct B-cell lineage (B1) whereas most other B-cell neoplasias derive from B2 lineage cells.[48,59-61] We do not know whether A-myb is expressed in the rare normal B1 cells and therefore whether A-myb expression reflects the phenotype of their normal counterpart. Normal and neoplastic B1 cells can be $CD5^+$ or $CD5^-$.[48,59-61] A-myb expression in CLL did not correlate with the expression of CD5 or other markers such as CD23, CD38, or CD39. The heterogeneity of expression in CLL did not correlate either with clinical parameters such as stage of the disease. Although the role of A-myb expression in CLL remains unclear, it provides a new marker of heterogeneity for this leukemia. In addition, our data confirm the hypothesis that A-myb expression is specific for a restricted stage(s) of B-cell differentiation rather than associated to a high proliferation rate because CLL cells are rather slow growing.[48,59]

We have also been able to study progression of a CLL case into RS in a patient whose PB contained both cell populations distinguishable on the basis of size and phenotype. The Richter's cells were likely to be derived from the original CLL cells, as described for most other Richter's cases,[49-54] because both carried the λ light-chain isotype. Progression was accompanied by a loss of CD5 expression and by an approximately 25-fold decrease in A-myb expression. Thus, once again the change in A-myb expression in this case did not correlate with proliferation but with a shift in differentiation. Studies aimed at defining the biologic function of the A-myb transcription factor in B lymphocytes will be necessary to understand its role in the neoplasms studied here.

C-myb and B-myb showed a quite different pattern of expression. They were expressed in most cell lines of all neoplastic groups, although their expression was somewhat heterogeneous within each group. Among fresh cases, c-myb and B-myb were again expressed in most ALL samples, whatever their subtype. On the contrary, only 3 of 20 CLL showed some c-myb expression and none had detectable B-myb. These data are in agreement with previous data, suggesting that in mature lymphoid cells, c-myb and B-myb expression correlates with proliferation,[18,19,62] because CLL usually have a low mitotic index.[48] Interestingly, c-myb was expressed even in very mature (myeloma) cell lines, indicating that this gene is expressed throughout B-cell differentiation, although generally at lower levels in mature cells (eg, myelomas, EBV-LCL, BL) than in immature ones (null and common ALL), as suggested previously for murine B-cell differentiation.[31] However, in the latter case the decrease in c-myb expression from pre-B to plasmacytoma lines appeared to be more consistent than that observed here and the c-myb mRNA was hardly detectable in the murine plasmacytoma cell lines.[31] On the other hand, we could easily detect c-myb mRNA expression in the mature human lines and in a few cases even at quite high levels (immunoblastic lymphomas), confirming that c-myb expression is not only related to differentiation. Of note is that no evidence of gross structural abnormality of c-myb or B-myb could be detected in the different leukemic samples studied.

We conclude that A-myb expression in neoplastic B cells is distinct and more restricted than that of c-myb and B-myb. In addition, our data clearly show that A-myb expression does not correlate with proliferation, because A-myb is absent from many rapidly proliferating cells (eg, ALL, RS, PLL) and on the contrary is present in cells with a low mitotic index (eg, some CLL). Similarly A-myb, unlike c-myb and B-myb, was not induced during mitogenic stimulation of normal B lymphocytes in vitro.[18,19] The very different pattern of expression of A-myb relative to c-myb and B-myb is interesting because these three myb proteins share several biochemical functions.[2,5,13,14,39] They all recognize the same DNA sequence and can activate transcription in different cell types, although A-myb appears to be the most active member of the family,[2,16] leading to the hypothesis that the different myb genes may have redundant roles. However, the specific pattern of expression of these genes, and in particular of A-myb, indicates that they have on the contrary distinct biologic functions in hematopoietic cells, perhaps through interaction with different sets of partners.[63,64]

Therefore, the lack of correlation between A-myb expression and proliferation in human B cells points to a role of A-myb in one or more of the differentiation processes taking place in B lymphocytes. A-myb expression has also been studied in *Xenopus* and in the developing mouse.[21,65] In *Xenopus*, A-myb appears to be most highly expressed in immature proliferating spermatogonia and ovarian tissue.[65] In the mouse, A-myb is restricted to mitotically active cells of the developing central nervous system, adult testis, and in B lymphocytes of germinal centers.[21] A-myb in the latter systems was hypothesized to be involved in the control of a proliferation and/or differentiation step preceding final differentiation in several cell types.[21] The data presented here point to a role of A-myb in B-lymphocyte differentiation. Because deregulation of differentiation is commonly observed in cancer, further study of the role of A-myb in the biology and transformation of the B-cell neoplasias presented here is warranted. Stable B-cell transfectants of A-myb are being generated to investigate this question further.

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