Expression of L-myc and N-myc Proto-oncogenes in Human Leukemias and Leukemia Cell Lines

By Harri Hirvonen, Veijo Hukkanen, Toivo T. Salmi, Tomi P. Mäkelä, Tarja-Terttu Pelliniemi, Sakari Knuutila, and Riitta Alitalo

The human myc family of proto-oncogenes consists of three well-characterized members, the c-myc, N-myc, and L-myc genes, which code for nuclear DNA-binding phosphoproteins. Accumulating evidence suggests that they have significant roles in the control of cell proliferation and differentiation. Deregulated expression of c-myc has been implicated in several human hematopoietic malignancies. We have studied the expression and mRNA processing of human L-myc, N-myc, and c-myc genes in a panel of human leukemias, leukemia cell lines, and normal hematopoietic cells. L-myc mRNA was expressed in three acute myeloid leukemias (AML) studied and in several myeloid leukemia cell lines. Only low expression levels were observed in adult bone marrow and in fetal spleen and thymus. The K562 and Dami leukemia cell lines showed a unique pattern of L-myc mRNA processing, with approximately 40% of L-myc mRNA lacking exon III and intron I. N-myc was expressed in five of six AML cases studied, in one of nine acute lymphocytic leukemia (ALL) cases, and in several leukemia cell lines. While c-myc mRNA was detected in all leukemias and leukemia cell lines studied, coexpression of all three myc genes was observed in Dami and MOLT-4 cell lines and in two AMLs, and either L-myc or N-myc was coexpressed with c-myc in several other cases. These results show that in addition to c-myc, the L-myc and N-myc genes are expressed in some human leukemias and leukemia cell lines, and suggest a lack of mutually exclusive cross-regulation of the myc genes in human leukemias.

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MATERIALS AND METHODS

Clinical specimens and fetal tissue samples. The fetal tissue samples were obtained from therapeutic abortuses at approximately 18 weeks of gestation. Leukemic cells were isolated from...
the peripheral blood or bone marrow of leukemia patients by Ficoll Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation before introduction of chemotherapy. Normal bone marrow polymorphonuclear and monocellular cells were isolated by Histopaque (Sigma, St Louis, MO) double-gradient centrifugation. Clinical data of the patients are given (see Table 1).

Cell culture. K562,36 U937,37 HL-60,38 MOLT-4,39 HEL,40 Jurkat,41 KG-1,42 ML-2,43 JOK-1,44 and RC-2A45 cells were grown in RPMI 1640 medium with 10% fetal calf serum (FCS), antibiotics, and L-glutamine. Dami cells46 were grown in Iscoves modified Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% horse serum. Induction of differentiation of K562 and HL-60 cells was commenced at a cell density of 3 to 5 x 10^6/mL with 1.6 to 3.2 nmol/L TPA (12-0-tetradecanoyl-phorbol-13-acetate, dissolved in DMSO). The SCLC cell lines U-1690th and GCL28 (a kind gift from Dr Charles Burns) were cultured in RPMI 1640 medium supplemented with 10% FCS and antibiotics.

Isolation of RNA and Northern analyses. RNAs from the fetal tissue specimens were extracted by the guanidine thiocyanate protocol44 and from the clinical specimens no. 1 to 10 by the lithium chloride method.45 From the cell lines and clinical specimens no. 11 to 17, poly(A) RNA was isolated by binding it directly to oligo(dT) cellulose as described.46 RNAs were isolated from freshly isolated cells except from the sample of patient no. 1, from which intact RNA was obtained after culturing the cells for 29 days in RPMI 1640 medium with 10% FCS and antibiotics. Fifteen-microgram aliquots of total cellular RNA were size-fractionated in 1% agarose formaldehyde gels and blotted onto nylon membranes (Genescreen Plus, DuPont, NEN, Boston, MA). Hybridizations and washes were performed under stringent conditions as suggested by the supplier.

Isolation and analysis of genomic DNA. To analyze myc gene copy number, genomic DNA of patients no. 1 to 10 was isolated by incubating the cells in 500 μg/mL proteinase K with 0.5% sodium dodecyl sulfate (SDS) for 4 to 5 hours, followed by extractions with phenol-chloroform and ethanol precipitation. DNA was removed by RNase A. Ten micrograms of genomic DNA was digested for 4 hours with the restriction enzymes EcoRI or SacI, run in 0.8% agarose gels, blotted onto GeneScreen Plus membrane and hybridized as suggested by the supplier.

Probes. The myc probe fragments are depicted in Fig 1. The c-myc probe was a 316-bp Clal/AI Al fragment of human c-myc exon III. This fragment was subcloned into C1al/BamHI sites of Bluescript KS+ (clone pCBS316) to allow antisense cRNA generation by T7 polymerase after XhoI linearization. The N-myc probe pN2bs349 was constructed by inserting the 349-bp XhoIl/PstI fragment (exon II) of the clone pNb-6' into BlueScript KS+, to allow antisense cRNA generation from T3 linearized pU313.blue is thus differentially protected by the different L-myc mRNAs. To generate an RB template clone pRb233bs, the 235-bp BglII/PstI fragment of RB cDNA clone RB5.8M(93)52 was inserted into the BamHI/PstI sites of BlueScript KS+, allowing antisense cRNA to be generated from the T3 promoter after linearization with SacI. The glyceraldehyde-3-phosphate dehydrogenase cDNA clone pHGAPDH13 was used as a reference probe in hybridizations with poly(A) RNA. For filter hybridizations, the specific fragments were isolated and purified from agarose gels by isochophoresis,44 and labeled using [32P]-dCTP (Amersham, Buckinghamshire, UK, no. PB.10205) according to the random priming method45 to specific activities of 1 to 2 x 10^6 cpm/μg. The GAPDH clone was labeled without isolating the specific cDNA fragment. After labeling, the probes were extracted with phenol-chloroform and purified through Sephadex G50 spin columns.

RNAse protection assays. For RNAse protection assays, the cRNA probes were generated from linearized templates (see above) using commercial transcription kits (Transprobe SP and T, Pharmacia) with [32P]-UTP (1,000 Ci/mmol, Amersham, no. PB.20383) as label nucleotide. The full-length antisense transcripts were eluted from 4.5% polyacrylamide gels and hybridized overnight to 15 μg of total cellular RNAs or 1.0 μg of poly-A+ RNAs in 80% formamide, 400 mmol/L NaCl, 40 mmol/L PIPES (piperazine-N,N’-bis[2-ethanesulfonic acid), pH 6.4, 1 μmol/L EDTA at 56°C. The L-myc (L2GEM450) and RB probes were mixed and applied as a probe cocktail to the hybridization reactions, the RB probe serving as an internal control. These probes were synthesized and purified separately to avoid possible contamination of the RB cRNA by prematurely terminated L-myc cRNA transcripts. The N-myc and c-myc cRNA probes (353- and 316-nt signals, respectively) were not mixed with the RB probe (322 nt), to avoid blurring of the signals by a residual RB probe observed in some experiments. The hybridizations were terminated by digestion with RNase A plus RNAse T, followed by proteinase K digestion, purified by phenol-chloroform extractions, ethanol precipitated, and electrophoresed in 4% polyacrylamide/8 mol urea gels with molecular weight ladders composed of end-labeled, MspI-generated fragments of pBR322.

RESULTS

L-myc is expressed in some myeloid leukemias. RNAse protection analyses using the probe pL2GEM450 resulted in strong L-myc signals in three of six acute myeloid leukemia (AML) samples studied (Fig 2A and Table 1). Much weaker protected bands were observed in RNAs from two lymphatic leukemias of class L2 (no. 7 and 9). These findings contrast with the lack of expression of L-myc mRNA in the other leukemic samples tested. The 457- and 421-nt bands are protected by L-myc mRNAs, which either contain or lack intron I, respectively. As an internal control, a human retinoblastoma cRNA probe was used in the same
Fig 2. L-myc expression in human leukemias and in leukemia cell lines. (A) RNase protection analysis of human leukemias and normal hematopoietic tissues with L-myc and RB antisense cRNA probes. The two protected fragments of 457 and 421 nt represent L-myc mRNAs, which either contain or lack intron 1, respectively, as a result of alternative mRNA processing. Strong signals are observed in three AML patients (no. 3, 5, and 6, types M2, M4, and M4, respectively). Weak L-myc signals are seen in normal bone marrow polymorphonuclear and mononuclear cells, as well as in fetal spleen and thymus. The RB cRNA probe was included in the same hybridizations as internal standard to verify the amount of RNA used. The 322-nt band results from residual undigested RB probe, and is seen also with the probe mix hybridized to carrier RNA (lane 3). The strong 233-nt RB signal is seen in all samples studied, but not with carrier RNA. (BM-polym/BM-monon., normal adult bone marrow polymorphonuclear/mononuclear cells.) The liver, spleen, and thymus samples are from a normal human second trimester fetus (see Materials and Methods). (B) RNase protection analysis of L-myc mRNA expression in human leukemia cell lines. Strong L-myc signals are observed in Dami, MOLT-4, U937, and K562 cells. The L-myc signal appears as a doublet, indicating the presence of both intron I splicing variants. The faint 322-nt band (indicated by *) is residual incompletely digested RB probe.
Table 1. Expression of c-myc, N-myc, L-myc, and RB Genes in Human Leukemias

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<th>Pt No.</th>
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<th>FAB Class</th>
<th>PB Leukocyte Count</th>
<th>Blasts (%)</th>
<th>Immunophenotype</th>
<th>Karyotype</th>
<th>c-myc</th>
<th>N-myc</th>
<th>L-myc</th>
<th>RB</th>
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<td>++</td>
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<td>36</td>
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<td>97</td>
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<td>46,XX, t(4;11)(q21;q23)</td>
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<td>-</td>
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<td>+</td>
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<tr>
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<td>CD13+, 14+, 33+, 2+ (50%), 7+, 11-, 20-, 36-</td>
<td>46,XY, t(15;17)</td>
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<td>+</td>
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<td>70</td>
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<td>46,XY/46, XY 3q-</td>
<td>+</td>
<td>~</td>
<td>~</td>
<td>-</td>
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</table>

Expression levels are expressed semiquantitatively as —, +, ++, or ++++. Note that the different myc mRNA levels cannot be compared with each other. In patient no. 2, c-myc expression was not evaluable due to lack of RNA for further analyses. Lack of some data for patients no. 8 (NIL-myc) and 9 (N-myc) is also due to paucity of RNA. Specimens no. 7 and 15 were obtained at relapse.

Abbreviations: FAB, French-American-British; PB, peripheral blood; ND, not done.

*The t(6;11) translocation of patient no. 3 is congenital.

K562 cells as compared with other cells and tissues analyzed (Fig 3B, right). In K562 and Dami cells, three protected fragments of 644, 602, and 505 nt were detected (Fig 3B). The major protected fragments of 602 and 505 nt correspond to the 3.6- and 2.0-kb mRNAs. The 2.0-kb mRNA appears unique to K562 and Dami cells. Indeed, approximately 40% of L-myc mRNA in K562 and Dami cells is of the 2.0-kb form, which lacks exon III and intron 1, in addition to the 3.8- and 3.6-kb L-myc mRNAs found in other cell types. The weaker 644-nt band corresponds to the 3.8-kb mRNA. In contrast, we observed virtually no signal corresponding to the 2.2-kb mRNA except in the U1690 cell RNAs. Figure 3 also demonstrates that in AML case no. 5 (M4), L-myc is expressed at several-fold higher levels than in human fetal brain (Fig 3B), which among normal tissues contains a very high amount of L-myc mRNA. These results are summarized in Fig 3C.

N-myc and c-myc expression in the leukemia cells. To analyze coexpression of the myc genes, we performed similar analyses for N-myc and c-myc mRNAs. For N-myc analyses, the cRNA template pN2bs349 was used. In RNase protection, five of six AML and one of nine acute lymphoid leukemias (ALL) showed strong N-myc signals (Fig 4A and data not shown). The N-myc signal intensities were of the same order of magnitude as in human fetal brain. A weak but definite N-myc signal was observed in fetal spleen, while fetal liver, fetal thymus, and adult bone marrow appeared devoid of N-myc mRNA (Fig 4A). Strong N-myc signals were obtained from MOLT-4, Dami, KG-1, Jurkat, and HEL cells, whereas U937, HL-60, K562, JOK-1, ML-2, and RC-2A cells contained no N-myc mRNA (Fig 4B).

Expression of c-myc was analyzed by Northern hybridization and RNase protection. The c-myc transcripts were detected in all leukemic cells, as well as in normal human hematopoietic tissues studied (data not shown). The highest c-myc mRNA levels did not correlate with lack of N-myc or L-myc expression in the leukemia specimens or in the cell lines (see Table 1). We found no amplifications of the myc genes in any of the patients studied by Southern hybridization (data not shown). The data on L-myc, N-myc, c-myc, and RB mRNA expression are summarized in Tables 1 and 2.

DISCUSSION

We have studied the expression of the c-myc, N-myc, and L-myc mRNAs in a panel of human leukemias, leukemia...
cell lines, and normal hematopoietic tissues, using RNase protection and Northern hybridization analyses. We show for the first time that in addition to c-myc, N-myc and L-myc are also expressed in some human leukemias and leukemia cell lines. High levels of L-myc mRNA were expressed in three cases of AML and in one ALL and three myeloid leukemia cell lines studied. The L-myc mRNA levels were comparable to those found in developing fetal brain, which contains high amounts of L-myc mRNA.\(^{28}\) Adult bone marrow polymorphonuclear and mononuclear cells contained very low amounts of L-myc mRNA, at levels similar to fetal spleen and thymus, while fetal liver appeared devoid of L-myc mRNA.

Since L-myc is expressed as four distinct mRNA species with different protein coding capacities, it is necessary to analyze the expression of each of the four transcript forms separately. Our approach in studying L-myc mRNA expression and structure enabled us to differentiate between the various structures of mature L-myc mRNAs, which were initially characterized in SCLC cell lines.\(^{29}\) These mRNAs differ from each other due to alternative splicing of intron I and alternative use of the intron II polyadenylation site.

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**Summary table**

<table>
<thead>
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<th>L-myc mRNAs</th>
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<td></td>
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</tr>
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<td>3.8 kb</td>
<td>III</td>
</tr>
<tr>
<td>3.6 kb</td>
<td>III/III</td>
</tr>
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<td>III/III</td>
</tr>
<tr>
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**Fig 3.** Unique processing pattern of L-myc mRNAs in K562 and Dam cells. (A) Structure of the human L-myc gene, the four alternatively processed L-myc mRNAs, and the cRNA template clone pU313.blue. The probe covers 42 bp of intron I, entire exon II (505 bp), exon II/exon III splice junction, and 97 bp of exon III. The four known L-myc mRNAs of 2.0, 2.2, 3.6, and 3.8 kb protect 505-, 547-, 602-, and 644-nt cRNA fragments, respectively. Exon II coding region; exon III coding region. (B) RNase protection analysis of L-myc mRNA structure. The 736-nt band represents residual undigested probe. The 644- and 602-nt bands correspond to the 3.8-kb and 3.6-kb mRNAs, respectively. Virtually no 2.2-kb mRNA is seen in any cells analyzed except in U1690. The lung adenocarcinoma cell line GLC28 results in a single protected band at 602 nt. This is due to a translocation of a novel gene (rif gene, Makela et al, 1991) 5' of the L-myc gene and a resulting fusion mRNA, which uses exclusively the L-myc exon II splice acceptor and splices out L-myc intron II. Note that a 505-nt fragment is protected only in Dam and K562 cells. It corresponds to the shortest (2.0 kb) L-myc mRNA that lacks intron I and exon III sequences. Densitometric scanning of the autoradiograms showed that approximately 40% of L-myc mRNA in these cells is of the 2.0-kb form. (C) Summary of the alternatively processed L-myc mRNAs in human leukemia cells, fetal brain, and the SCLC cell line U1690. The relative amounts of the different L-myc mRNA forms are expressed semiquantitatively as −, +, ++, or ++++, with respect to each other.
This results in the 3.8-, 3.6-, 2.2-, and 2.0-kb L-myc mRNAs (see Fig 3A). The long form mRNAs (3.6 and 3.8 kb) code for a full-length L-myc protein. The short form L-myc mRNAs (2.0 and 2.2 kb) lack exon III. Thus, they have the capacity to code for a short form L-myc protein whose carboxyl terminus extends 41 amino acids into intron II. Indeed, such short L-myc proteins have been demonstrated in SCLC cell lines with amplified and overexpressed L-myc genes. The majority of L-myc mRNA in normal human cells or tissues is of the 3.6- and 3.8-kb forms. Interestingly, two leukemia cell lines (K562 and Dami) showed a unique pattern of L-myc mRNA processing: approximately 40% of total L-myc mRNA in K562 and Dami cells was of the 2.0-kb form, which may represent a tumor cell-specific alteration of L-myc mRNA processing in these leukemia cells. Virtually no 2.2-kb L-myc mRNA was detected, except in the U1690 SCLC cells, which have a 40-fold amplified L-myc gene. The short L-myc mRNA in K562 and Dami is of interest due to its different protein coding capacity. We are currently attempting to determine whether a truncated L-myc protein is translated from the short L-myc transcripts in these cells. However, current detection methods are not sensitive enough for such analyses; the levels of L-myc mRNA in these cells are very low when compared with SCLC cells with amplified L-myc genes, which have been used in all L-myc protein analyses so far.

RNAse protection analyses showed high N-myc mRNA levels in three AML patients (no. 3, 6, 11) and one T-cell ALL patient (no. 10), as well as in Dami, KG-1, MOLT-4, Jurkat, and HEL cell lines. These findings contrast with the lack of N-myc expression in normal hematopoietic cells and tissues, where only fetal spleen yielded a weak N-myc signal. Thus, one could speculate that N-myc expression in leukemia cells is not an event secondary to transformation, but may contribute directly to the malignant phenotype. However, the normal hematopoietic specimens studied here represent mixed cell populations. There may exist a minor subpopulation of N-myc expressing cells in normal bone marrow, but its N-myc mRNA may be diluted beyond detection by RNA from N-myc negative cells. Similarly, the low level of L-myc mRNA expression observed, eg, in bone marrow, may also result from as yet unidentified subpopulation(s) of hematopoietic precursor cells with higher L-myc expression levels.

Table 2. Expression of c-myc, N-myc, L-myc, and RB Genes in Human Leukemia Cell Lines and Normal Hematopoietic Tissues

<table>
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<tr>
<th>Cell Line/Tissue</th>
<th>c-myc</th>
<th>N-myc</th>
<th>L-myc</th>
<th>RB</th>
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<td></td>
<td>-------</td>
<td>------</td>
</tr>
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<td>++++</td>
<td>++</td>
<td>++++</td>
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<td>++++</td>
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<tr>
<td>Fetal thymus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Expression levels are expressed semiquantitatively as -, +, ++, or ++++. Note that mRNA levels for the different myc genes cannot be compared with each other. The c-myc mRNA analyses were performed by Northern hybridization; L-myc, RB, and N-myc mRNA analyses by RNAse protections.

*The regenerating bone marrow specimen was obtained from an otherwise healthy child during recovery from neutropenia associated with viral infection.
mRNA levels. However, we consider it unlikely that RNA from the normal peripheral blood cells of, eg, patient no. 3 with a low percentage of blasts (34%), could contribute to the intense L-myc signal obtained in this case, as we only observe a barely detectable L-myc signal in RNase protection analyses of normal peripheral blood (H. Hirvonen, unpublished observations).

N-myc and L-myc mRNAs were expressed only in combination with c-myc mRNA, which was readily detected in all specimens (see Table 1). This observation is of interest, as negative cross-regulation of myc genes has been shown in several experimental systems with abundant myc expression. It suggests that at lower expression levels, such negative cross-regulation may not be manifested, since we detected coexpression of two to three myc genes in several types of leukemia cells (Table 1). Further investigations are required to find out whether the combinatorial expression of myc genes has a role in the growth regulation and/or malignant transformation of hematopoietic cells.

It should be emphasized that we did not observe any distinct clinical characteristics associated with L-myc or N-myc expression in this small set of patients. Larger clinical materials and follow-up studies are therefore required to establish possible clinical correlates and significance of L-myc and N-myc expression in human leukemias. The observation of L-myc expression in three AML cases, as well as in three myeloid and one ALL cell line, also warrants further studies on the possible role of L-myc in hematopoietic cell growth and differentiation. Experimental evidence suggesting a role for L-myc in transformation of myeloid cells has been obtained from transgenic mice; Epither-L-myc transgenic mice show perturbation of myeloid cell development and develop myelomonocytic malignancies at high frequency, but after long latency periods.57

In normal cells, c-myc expression is under negative feedback regulation at the level of transcriptional initiation. This negative autoregulation is frequently lost in malignant cells.60 However, possible negative autoregulation mechanisms of N-myc and L-myc and their eventual failure in malignant cells remain to be established. These questions must be elucidated in order to distinguish whether N-myc and L-myc expression in leukemic cells results from proo-primo enhanced expression, or whether activation of these genes occurs secondary to differentiation arrest. Further mapping of N-myc and L-myc expression to eventually distinct hematopoietic precursor cells and developmental stages will undoubtedly advance our understanding of the roles of myc genes in hematopoietic cell growth and differentiation.

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Expression of L-myc and N-myc proto-oncogenes in human leukemias and leukemia cell lines

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