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Differential Expression of the Oncoproteins c-myc and c-myb in Human Lymphoproliferative Disorders

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Altered regulation of oncogene expression has been described in a variety of hematopoietic malignancies. In this study we analyzed the protein level of c-myc and c-myb in 15 established cell lines derived from lymphopoietic disorders and in 45 samples from patients with acute or chronic lymphatic leukemias. Oncoproteins were assayed by radioimmuno-precipitation with polyclonal rabbit antibodies. In B-cell derived lines, such as Burkitt lymphoma and plasmocytoma lines, we found high amounts of c-myc and no or low amounts of c-myb. In contrast, all T-cell-derived lines revealed high levels of c-myb. In addition, T-lymphoma cell lines of low malignancy also exhibited high levels of c-myc, while T-cell lines of high malignancy (acute T-lymphoblastic leukemias) exhibited moderate levels of c-myc. Of the 45 patient samples analyzed, only three (one B-prolymphocytic and two acute T-lymphoblastic leukemias) contained detectable amounts of myc or myb protein. Corresponding to the results found in established cell lines, the B-cell sample revealed a high level of c-myc but no c-myb, while the T-cell samples revealed high levels of c-myb and no or low levels of c-myc. We therefore conclude that the predominance of c-myc or c-myb expression in malignant lymphoproliferative disorders may be associated to the B-cell or T-cell lineage, respectively. Further, regarding the T-cell lines, there is a possible correlation between cell maturation and the level of c-myc found together with a consistently elevated c-myb.
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INTRODUCTION

THE proto-oncogenes c-myc and c-myb are involved in regulatory processes that control cell proliferation and differentiation. For example, c-myc and c-myb mRNA increase transiently after stimulating quiescent cells to proliferate. C-myb mRNA reaches its maximum level in the late G1 and S phase of each cell cycle. Besides the association of high myb mRNA levels with the cell cycle, there appears to be a tissue-specific high level of myb in immature thymocytes, which disappears after differentiation has been induced. Regarding the myc proto-oncogene, altered expression has been described as a characteristic feature of a variety of B-cell neoplasias. It has been shown that altered myc expression blocks differentiation at an early stage of B-cell development and predisposes to progress to a neoplastic phenotype [1, 2].

Under the assumption that the activation of c-myc and c-myb oncogenes might be related to certain subtypes of hematopoietic neoplasias and might therefore give insight into their prognosis, several groups have analyzed leukemia cell lines and leukemia cell samples from patients for the presence of c-myc or c-myb mRNA. Except for Burkitt lymphomas, which showed a strong activation of c-myc throughout, the expression of c-myc and c-myb was so variable that no clearcut correlation with leukemia subtypes could be made [3–10].

Recently, more systematic investigations showed high levels of myc and low levels of myb mRNA in acute lymphoblastic leukemias of B cell type (B-ALL), low levels of myc and high levels of myb-mRNA in pre B-ALL, and moderately elevated levels of myc and myb mRNA in T-ALL [11]. Elevated transcript levels of c-fos, c-fms and c-fes were detected in acute myeloblastic leukemia (AML). The oncoprotein c-fos was almost exclusively restricted to the myelomonocytic (FAB M4) or monocytic (FAB M5) subtype of AML [11, 12]. In multiple myeloma Selvanayagam *et al.* detected elevated c-myc mRNA levels in nine of 36 bone marrow samples analyzed [13].

It is nevertheless doubtful whether gene activation can be safely assessed by the analysis of mRNA alone, because the amount of mRNA which will be detected depends on the rates of synthesis and degradation, both underlying different mechanisms of regulation. Thus we cannot reliably deduce from the amount of mRNA detected, which amount will be translated into the protein product and if this translation product can function normally.

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Since proteins are the relevant effector molecules in the cascade of expression, we set out to study the c-myc and c-myb protein expression in leukemia cells. Our aim was to try to detect specific patterns of c-myc and c-myb expression characteristic for certain leukemia/lymphoma subtypes. We utilized immunoprecipitation of radiolabeled cell extracts with polyclonal rabbit antisera. This assay system permits the simultaneous detection of both oncoproteins in a given cell lysate.

MATERIALS AND METHODS

The cell lines used were obtained from the American Type Culture Collection (ATCC). They were grown under standard conditions. KG-1 and KG-1a were cultured in IMDM with 20% fetal calf serum (FCS), the remaining cell lines in RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin. We analyzed four T-ALL lines (Molt 4, Jurkat, Reh, CCRF-CEM), three T-non-Hodgkin lymphoma lines (HUT 78, K37, H9) and five Burkitt lymphoma lines (Raji, Jiyoye, HRIK, Namalva, Daudi) and three plasmocytoma lines (U266, RPMI 1788, RPMI 8224).

Leukemia cell specimens

Leukemia cells were isolated from the peripheral blood of patients by Ficoll density gradient centrifugation. The purity of the resulting leukemia cell preparations was greater than 95%. In patients with chronic myeloid leukemia in chronic phase we isolated leukemia cells from the buffy coat of peripheral blood.

Metabolic protein labeling

1×10^7 cells were washed with phosphate-buffered saline (PBS) and suspended in 400 µl labelling medium containing methionine-free RPMI 1640, 5% dialyzed fetal calf serum and 300 µCi[^{35}S]methionine. The cell suspension was incubated for 90 min at 37°C in a 5% CO_2 atmosphere.

Radioimmunoprecipitation

1×10^7[^{35}S]methionine labelled cells were washed with PBS, suspended in 1500 µl lysis buffer (10 mM Tris pH 7.5, 50 mM NaCl, 0.5% NP40, 0.5% Na-desoxycholate, 0.5% Na-dodecylsulfate) and sonified three times for 2 s at 100 W. Undissolved material was removed by centrifugation (15,000 *g*, 4°C, 30 min). Lysates were preabsorbed with normal rabbit serum (30 min, 0°C) and the resulting immune complexes were removed by binding to *Staph. aureus* (Pansorbin, Calbiochem). 10 µl of polyclonal rabbit anti-hu-c-myc or anti-hu-c-myb were added to 300 µl cell lysate containing a TCA precipitable radioactivity of 10×10^6 cpm. After incubating for 60 min at 0°C, 30 µl *Staph. aureus* was added and incubated for another 30 min at 0°C. The bacteria carrying the immune complexes were washed three times in 500 µl RIPA buffer [50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 1% Triton X-100, 1% Na-desoxycholate, 0.1% Na-dodecylsulfate], once in a buffer containing 10 mM Tris–HCl (pH 7.2), 1 M NaCl and 0.1% NP-40 and finally once in 50 mM Tris–HCl (pH 7.2). The pelletted bacteria carrying immune complexes were dissolved in 40 µl sample buffer (Laemmli). Protein samples were electrophoresed in 10% SDS-polyacrylamide gels. The gels were dried and autoradiographed for 1–2 weeks.

Antibodies

Polyclonal antibodies against hu-c-myc were raised in rabbits against a bacterially expressed fusion protein MS2-hu-c-myc [15]. Polyclonal antibodies against hu-c-myb were prepared by immunizing rabbits with bacterially expressed v-myb [16].

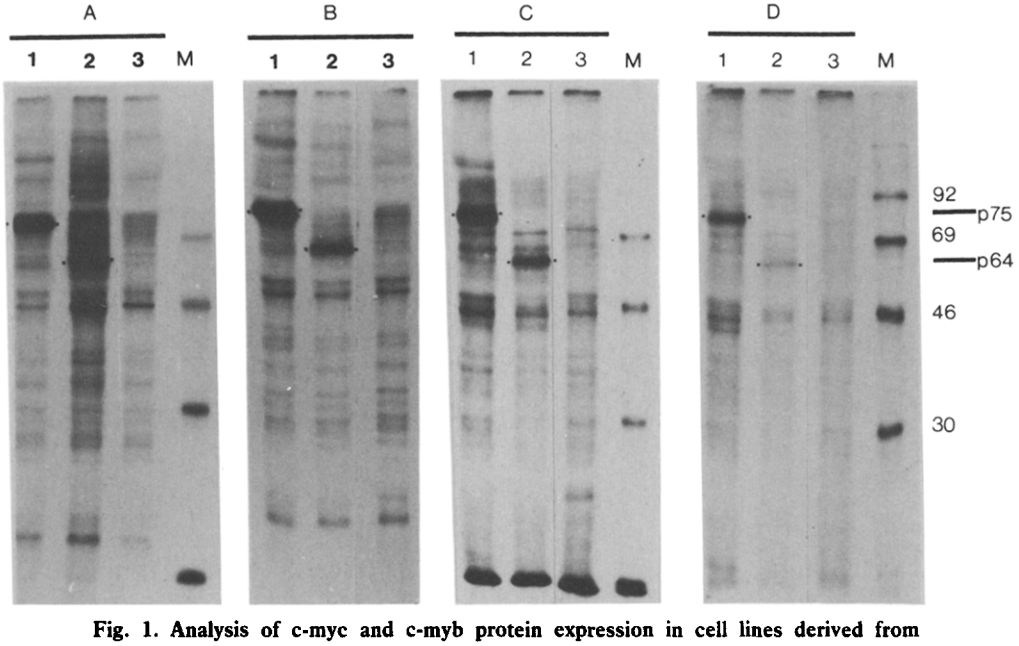

Fig. 1. Analysis of c-myc and c-myb protein expression in cell lines derived from acute T-lymphoblastic leukemias by immunoprecipitation (A = Reh, B = CCRF-CEM, C = Molt4, D = Jurkat). Cells were labelled metabolically with [^{35}S]methionine for 90 min, lyzed and treated with rabbit antiserum against c-myb (1), c-myc (2) and normal rabbit serum (3). Subsequently, a *Staphylococcus aureus* suspension was added. The precipitates were electrophoretically analyzed on 10% SDS-polyacrylamide gels and exposed for autoradiography for 1–2 weeks. M indicates the lane with the molecular weight reference proteins. p75 hu-c-myb, p64 hu-c-myc.

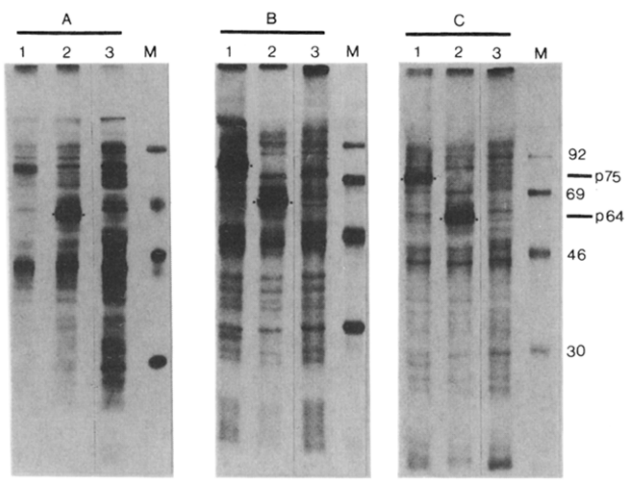

Fig. 2. Analysis of c-myc and c-myb protein expression in cell lines derived from T-cell non-Hodgkin lymphomas of low malignancy (A = HUT 78, B = K 37, C = H 9). Cells were treated as described in Fig. 1. p75 hu-c-myb, p64 hu-c-myc.

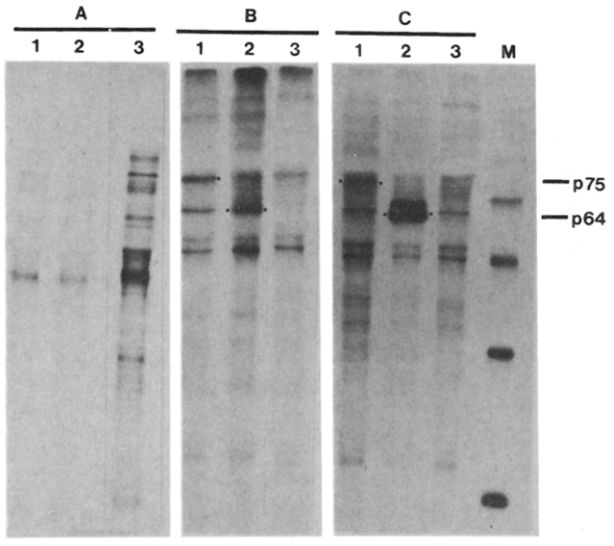

Fig. 4. Analysis of c-myc and c-myb protein expression in cell lines derived from human plasmocytomas (A = U 266, B = RPMI 1788, C = RPMI 8224). Cells were treated as described in Fig. 1.

Antibodies were a gift from K. Moelling, Max Planck-Institut für Molekulare Genetik, Berlin.

RESULTS

c-myc and c-myb protein expression in lymphopoietic cell lines

Fifteen cell lines derived from acute leukemias and lymphoproliferate disorders were analyzed for the simultaneous expression of c-myc and c-myb protein. Lysates of cells metabolically labeled with [^{35}S]methionine were subjected to indirect immunoprecipitation with polyclonal rabbit antibodies against p64 hu-c-myc and p75 hu-c-myb. Figures 1–4 show the autoradiograms obtained after separation of immunoprecipitates on SDS-polyacrylamide gels. Lines derived from acute T-lymphoblastic leukemias (T-ALL), such as Molt 4, Jurkat, Reh and CCRF-CEM show a high expression of hu-c-myb and a comparatively lower expression of hu-c-myc (Fig. 1). Cell lines derived from T-cell non-Hodgkin lymphomas of low malignancy, such as K37 and H9, show an equally high expression of both c-myc and c-myb. HUT 78 expresses c-myb only to a lower extent (Fig. 2). Regarding cell lines derived from B-cell lymphoproliferative disorders, we analyzed five Burkitt lymphoma lines (HRIK, Namalwa, Jiyoye, Raji, Daudi) and three plasmocytoma lines (U 266, RPMI 8226, RPMI 1788). Both, the Burkitt lymphoma and the plasmocytoma lines exhibited a high expression of p64 hu-c-myc and only a low or undetectable expression of p75 hu-c-myb (Figs 3 and 4).

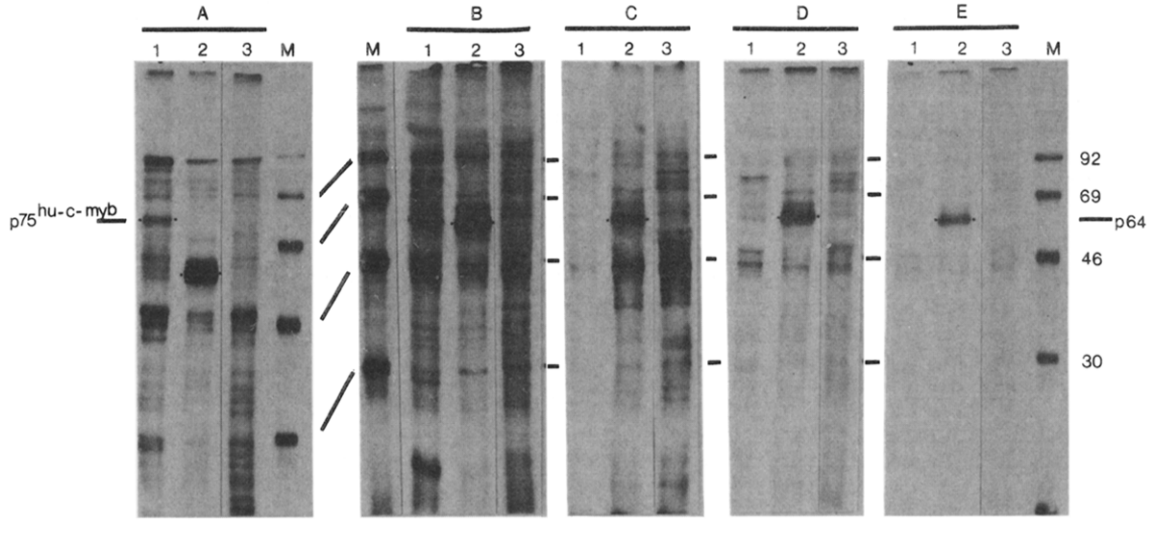

Fig. 3. Analysis of c-myc and c-myb protein expression in cell lines derived from Burkitt lymphomas (A = Raji, B = Jiyoye, C = HRIK, D = Namalwa, E = Daudi). Cells were treated as described in Fig. 1. p64 hu-c-myc.

Table 1. Expression of the oncoproteins c-myb and c-myc in cell lines derived from human lymphatic neoplasias

	c-myb	c-myc
Molt 4	+++	+
Jurkat	++	+
Reh	+++	++
CCRF-CEM	+++	++
K 37	+++	+++
H 9	+++	+++
HUT 78	+	+++
HRIK	(+)	++
Namalwa	(+)	+++
Jiyoye	(+)	+++
Raji	+	+++
Daudi	(+)	++
U 266	–	–
RPNI 8226	(+)	+++
RPMI 1788	+	++

(–) – (+++): arbitrary scale of oncoprotein expression.

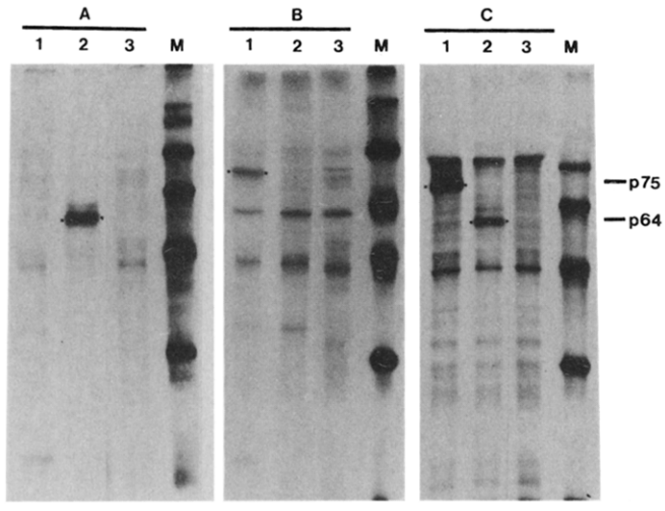

Fig. 5. Analysis of c-myc and c-myb protein expression in cells from a patient with B-prolymphocytic leukemia (A) and two patients with acute T-lymphoblastic leukemias (B, C). Cells were treated as described in Fig. 1.

The relative levels of c-myc and c-myb protein present in each cell line were graded on a scale from (–) to (+++) and are shown in Table 1. The comparison of the different cell lines with each other reveals similar patterns of oncoprotein expression within each diagnostic category of lymphoproliferative disorders and that the patterns differ characteristically between the different diagnostic groups. Cell lines belonging to the T-cell lineage show a high expression of c-myb. T-cell lines derived from T-non-Hodgkin lymphomas also exhibit a high expression of c-myc, while cell lines derived from T-ALLs only show a moderate c-myc expression. Cell lines of B-cell origin, such as Burkitt lymphoma and plasmocytoma cell lines exhibit a high expression of c-myc and a low or undetectable expression of c-myb.

Table 2. Expression of the oncoproteins c-myb and c-myc in human lymphatic leukemias

	N =	Positive	c-myb	c-myc
CLL	29	0		
IC	1	0		
HCL	1	0		
P-CLL	1	1	–	+++
T-ALL	13	2	+++	+
			++	–

CLL = chronic lymphatic leukemia, IC = lymphoplasmocytoid immunocytoma, HCL = hairy cell leukemia, P-CLL = prolymphocytic leukemia, ALL = acute lymphoblastic leukemia. (–) – (+++): arbitrary scale of oncoprotein expression.

c-myc and c-myb protein expression in leukemia cell specimens from patients

We analyzed 45 cell samples obtained from the peripheral blood of patients with leukemia. Cells were subjected to identical labelling and immuno-precipitation procedures as cell lines. The disorders studied and the results are displayed in Table 2. Surprisingly, we could only detect c-myc and c-myb protein expression in cells from three patients. In one leukemia cell sample derived from a patient with prolymphocytic leukemia high amounts of c-myc were detected while c-myb remained undetectable. In two of 13 leukemia cell samples from patients with acute T-lymphoblastic leukemias we found high amounts of c-myb and no or only low amounts of c-myc protein (Fig. 5).

DISCUSSION

In this study we wished to associate specific patterns of c-myc and c-myb protein expression with subtypes of lymphatic leukemias. As opposed to most of the previous work published, we analyzed the oncoproteins, because we thought that they would tell us more about the actual status of oncogene activation than analysis of the corresponding mRNA.

We found lineage specific patterns of differential c-myc and/or c-myb protein expression. Cell lines of B-cell origin, such as Burkitt lymphoma and plasmocytoma lines, contain high levels of p64 hu-c-myc and very low or undetectable amounts of p75 hu-c-myb. In contrast, cell lines of T cell origin show high levels of c-myb protein. The amount of c-myc protein is lower in relation to myb in T-ALL lines, and high in three lines derived from T-non-Hodgkin lymphomas of low malignancy. The analysis of three patient cell samples was similar. Oncoprotein expression in a cell sample from a patient with B-prolymphocytic leukemia, derived from mature B lymphocytes, resembled the pattern found in plasmocytoma cell lines. Both show a high content of p64 c-myc and no detectable p75 c-myb. Further, in accordance with our findings in established T-ALL lines, two T-ALL patient cell samples exhibited high levels of myb

accompanied by undetectable myc levels, in one case, and low myc levels in the other case.

The evaluation of our data in comparison with work published earlier is difficult, because so far most of the work is based on mRNA analysis. Altogether, these data do not hint at a possible correlation of specific patterns of myc and/or myb mRNA expression to certain leukemia subtypes. Ferrari *et al.* found elevated c-myb mRNA in five of six and elevated c-myc mRNA in four of six ALLs. Four of six samples from chronic lymphatic leukemia (CLL) contained elevated myc mRNA [16]. An analysis of oncogene mRNA expression by Mavilio *et al.* showed in preB-ALL cells high levels of myb and no myc [11]. Conversely, in B-ALL myc could be demonstrated but no myb. Moderate levels of both myc and myb were present in T-ALLs. In a similar study, Blick *et al.* detected elevated mRNA levels for myc in three of three ALL specimens and in three of three CLL specimens [8]. Myb was not detectable in five CLL samples tested, but it was present in three of four ALL samples. All these three samples positive for myb were T-ALLs, the negative sample was from a B-ALL. Evidence for the involvement of the myc oncogene in the pathogenesis of human plasmocytoma stems from a detailed study by Selvanayagam *et al.* [13]. They detected an overexpression of myc mRNA in nine of 36 plasmocytoma cell samples from patients, associated with a myc gene rearrangement in two cases.

When judging these data, one has to consider the limitations of mRNA analysis. Preisler *et al.* present data that illustrate this problem [17]. A simultaneous analysis of myc mRNA and myc protein in leukemia cells demonstrated that gene expression judged by their mRNA level is not in all cases reflective of the expression at the protein level. This is due to independent regulatory processes for transcription, translation or posttranslational control.

The myb protein expression in human lymphoid malignancies was recently analyzed in a detailed study by Bading *et al.* [18]. They utilized an immunohistochemical staining method on lymph node sections, which enables the detection of single antigen positive cells. They conclude that myb protein is expressed at high levels in disorders derived from immature lymphatic precursors with high proliferative activity, and that it is absent in lymphomas of intermediate and low malignancy. They do not find a correlation of myb expression with the B or T lineage. In our view the interpretation of their data is difficult because the percentage of antigen-positive cells in lymph nodes that are defined as myb positive varies greatly—between 1% and 80%. Moreover, in sections with low numbers of myb-positive cells it is difficult to ascertain whether a positively reacting cell really belongs to a malignant cell clone. On the basis of our data we come to a somewhat divergent conclusion. This may in part be due to our different approach, which measures average antigen levels in a cell population and therefore has a lower sensitivity. However, our system has the advantage that it is dealing with homogeneous, almost pure (>95%) tumor cell populations. Further, it permits the simultaneous detection of both myb and myc antigens.

In the avian system, several lines of evidence demonstrate the relationship between the deregulated expression of c-myc and the development of bursal cell derived lymphomas [1]. Also, in the transgenic mouse model, high constitutive expression of the inserted c-myc oncogene coupled to the immunoglobulin enhancer leads to the development of B cell lymphomas at a high frequency [19]. Beside an increased c-myb expression in proliferating hematopoietic and non-hematopoietic cells, c-myb is expressed at a high level in immature thymic lymphocytes [2]. In conclusion, our observations are in agreement with the concept deduced from results in experimental animals.

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