MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to *BCL2*

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ABSTRACT During their lifespan, immature cells normally pass through sequential transitions to a differentiated state and eventually undergo cell death. This progression is aberrant in cancer, although the transition to differentiation can be reestablished in inducible leukemia cell lines. This report describes a gene, *MCL1*, that we isolated from the ML-1 human myeloid leukemia cell line during phorbol ester-induced differentiation along the monocyte/macrophage pathway. Our results demonstrate that expression of *MCL1* increases early in the induction, or "programming," of differentiation in ML-1 (at 1–3 hr), before the appearance of differentiation markers and mature morphology (at 1–3 days). They further show that *MCL1* has sequence similarity to *BCL2*, a gene involved in normal lymphoid development and in lymphomas with the t(14;18) chromosome translocation. *MCL1* and *BCL2* do not fall into previously known gene families. *BCL2* differs from many oncogenes in that it inhibits programmed cell death, promoting viability rather than proliferation; this parallels the association of *MCL1* with the programming of differentiation and concomitant maintenance of viability but not proliferation. Thus, in contrast to proliferation-associated genes, expression of *MCL1* and *BCL2* relates to the programming of differentiation and cell viability/death. The discovery of *MCL1* broadens our perspective on an emerging *MCL1*/*BCL2* gene family and will allow further comparison with oncogene families.

Normal tissues are characterized by a balance between cell proliferation, cell differentiation, and cell death. The molecular programs that control this balance are aberrant in cancer. Gaining an understanding of these programs involves identifying genes that influence proliferation and the transitions to differentiation and death (e.g., oncogenes, tumor-suppressor genes). Our research focuses on the molecular events that underlie the transition from proliferation to differentiation in a hematopoietic cell line, the ML-1 human myeloid leukemia cell line (1–3). ML-1 cells proliferate as immature myeloblasts and can be induced to differentiate to monocytes/macrophages with phorbol 12-myristate 13-acetate ["12-*O*-tetradecanoylphorbol 13-acetate" (TPA)] (1–3). The differentiated cells lose proliferative capacity and accumulate in the G_0/G_1 phase of the cell cycle, while remaining viable and capable of carrying out normal monocyte/macrophage functions (1–3). In sum, immature, proliferative cells convert to a differentiated, viable, nonproliferative phenotype.

In ML-1 cells, the initial induction or "programming" of this conversion can be separated from the ensuing phenotypic transition: upon exposure to TPA under optimal conditions, ML-1 cells become induced (i.e., irreversibly committed, or programmed) at ≈3 hr and then undergo phenotypic differentiation at 1–3 days (1–3). The initial induction probably involves the combined action of a variety of genes, some increasing and others decreasing in expression. Previous work showed the c-*myb* oncogene mRNA to decrease within

3 hr [i.e., during induction and before loss of DNA synthesis or overt differentiation (2, 3)]. In the work described here, we set out to identify genes that increase in expression within this early time frame. The p53 tumor-suppressor gene product had previously been shown (4) to increase in differentiation, but this occurred at later times (≥1 day). Our rationale in seeking mRNAs increased within 3 hr was that (*i*) some of these "early-induction" genes might influence later phenotypic changes and (*ii*) a similar strategy had been successful in identifying interesting "early-response" genes expressed upon the stimulation of cell proliferation (5).

We describe here an early-induction gene, *MCL1*†, isolated from maturing ML-1 myeloid cell leukemia. We found *MCL1* to have sequence similarity to *BCL2*, a gene that was originally identified in B-cell lymphomas. Most follicular B-cell lymphomas have a t(14;18) chromosome translocation that juxtaposes *BCL2* with the immunoglobulin heavy-chain locus; this results in deregulated expression of an unaltered *BCL2* gene product (6–9). *BCL2* had not previously been found to have sequence similarity to known cellular oncogenes; it does not contain motifs characteristic of other gene families, although it does have distant similarity to a viral gene, BHRF1 (8, 10, 11). Expression of *BCL2* relates to inhibition of programmed cell death (12–14), while that of BHRF1 occurs early in the lytic cycle of Epstein–Barr virus (10, 11). *MCL1* provides an example of a cellular gene related to *BCL2*. This allows us to begin to define the characteristics of what appears to be a gene family represented by *MCL1*, *BCL2*, and BHRF1.

MATERIALS AND METHODS

Preparation of a cDNA Library from TPA-Induced ML-1 Cells. ML-1 cells (1–3) were induced with TPA by a protocol that optimizes the programming of differentiation (3): cells were exposed to 0.5 nM TPA for 3 hr, after preincubation under reduced serum conditions for 3 days. Poly(A)⁺ RNA was isolated (2, 3) and used in oligo(dT)-primed first-strand cDNA synthesis, which was carried out with Moloney murine leukemia virus reverse transcriptase (BRL). After second-strand cDNA synthesis, the cDNA was treated with S1 nuclease (BRL), *Eco*RI methylase (New England Biolabs), and Klenow fragment of DNA polymerase (BRL). After addition of *Eco*RI linkers, cDNA of >500 bp was isolated (Bio-Gel A50M column, BioRad), cloned into the λgt10 vector, packaged (Packagene, Promega), and infected into *Escherichia coli* C600hfl. The yield was 3 × 10⁴ plaque-forming units/ng of cDNA, with an average insert size of 1.5 kb.

Screening for cDNAs Representing Early-Induction Genes. For differential screening, recombinant phage from the

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Abbreviation: TPA, "12-*O*-tetradecanoylphorbol 13-acetate" (phorbol 12-myristate 13-acetate).
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†The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession no. L08246).

cDNA library were plated and transferred to nitrocellulose filters. Replicate filters were hybridized to ^{32}P-labeled cDNA probes derived either from TPA-induced or uninduced cells. [Induction with TPA was carried out for 3 hr with the above-described optimized protocol. The cDNA probes were synthesized by reverse transcription of poly(A)$^+$ RNA, using random hexamers as primers.] Primary and secondary screenings were carried out to identify clones exhibiting preferential hybridization to the cDNA probe from induced cells. One of the positive clones obtained, dif8C, was subcloned into the pBluescript plasmid (Stratagene). Clone dif8C contained nucleotides 3150–3934 of *MCL1* followed by 11 adenine residues. This clone was used in obtaining additional cDNAs, both from the above cDNA library and from a cDNA library from TPA-induced U-937 cells (Clontech).

Characterization of the Early-Induction cDNAs Obtained by Differential Screening. Northern blotting (2, 3) was carried out using probes for *MCL1*, β-actin (15), c-*myb* [pCM8 from R. Dalla-Favera (16)], and CD11b [from D. G. Tenen (17)]. After densitometric scanning, the values for expression of *MCL1* (the more abundant, 3.8-kb *MCL1* transcript) were normalized by dividing by the corresponding value for β-actin. Relative expression of *MCL1* was then estimated as the ratio of expression in TPA-induced cells to that in uninduced controls.

Differentiation markers were monitored by flow cytometry on a FACScan using phycoerythrin-conjugated antibodies to CD11b and CD14 (Becton Dickenson). Corrected mean fluorescence intensity was calculated by subtraction of background, which was determined by using isotype-matched controls (IgG2a from Becton Dickinson and IgG2b from Coulter Immunology).

Sequencing was carried out by the dideoxy chain-termination method (18, 19) with a modified T7 polymerase (Sequenase, United States Biochemical). The *MCL1* coding region was sequenced on both strands, and the 3' untranslated region on one strand. Computer analysis was carried out using the Genetics Computer Group sequence-analysis software (Madison, WI), the PESTFIND program (from Martin Rechsteiner, University of Utah), and the SIGSEQ programs (from Jeffrey Gordon, Washington University).

RESULTS

Identification of a cDNA Clone Representing *MCL1*. To search for early-induction genes in ML-1 cells, we adopted the simple approach of differential screening: cells were exposed to TPA for 3 hr according to a protocol that optimizes the programming of differentiation (3). A cDNA library representing these programmed cells was screened by hybridization to two separate probes, one derived from the TPA-induced cells and the other from a parallel culture of uninduced cells. Upon screening of 30,000 plaques, we identified two cDNA clones that exhibited preferential hybridization to the probe from induced cells. One of these, dif8C, represented *MCL1*.

Time Course of Expression of *MCL1* During Myeloid Leukemia Cell Differentiation. Having identified *MCL1* as a candidate early-induction gene, we wished to characterize the relationship between its expression and other differentiation-related changes. Accordingly, Northern blotting was performed to monitor the *MCL1* mRNA at various times during the differentiation of ML-1 cells; representative blots were also stripped and assayed for expression of other mRNAs known to change during differentiation [e.g., *MYB* (2, 3)]. These blots confirmed that expression of *MCL1* was low in uninduced cells and increased dramatically early in induction with TPA (Fig. 1*A*, lanes 1 and 2). Upon induction for 3 hr, two mRNA transcripts were detected: a 3.8-kb transcript, which increased >6-fold, and a 2.5-kb transcript,
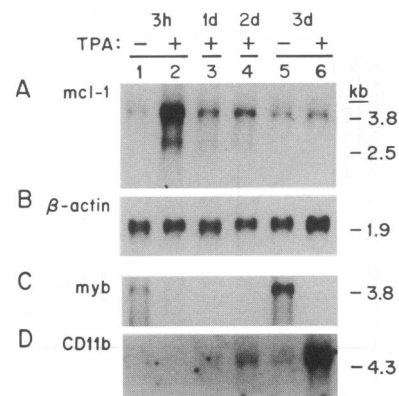

FIG. 1. Expression of *MCL1* in uninduced and TPA-induced ML-1 cells. Cells were exposed to 0.5 nM TPA and assayed for the *MCL1* (probe dif8C-p3.2, containing nucleotides 7–1484 of *MCL1*), β-actin, c-*myb*, and CD11b mRNAs at the indicated times; h, hours; d, days.

present at about one-third the level of the 3.8-kb transcript. The increase in *MCL1* mRNAs coincided with the decrease in *MYB* mRNA (Fig. 1*C*, lanes 1, 2, 5, and 6; refs. 2 and 3) and preceded any increase in the mRNA encoding the CD11b differentiation marker (Fig. 1*D*). A TPA-induced early increase in expression of *MCL1* was also seen in other cell lines of myelomonocytic origin or character [HL-60 promyelocytic leukemia, U-937 histiocytic lymphoma, and K-562 chronic myelogenous leukemia (data not shown)].

In further time-course experiments, we defined the relationship between expression of *MCL1* and the appearance of markers of phenotypic differentiation. We found that the increase in *MCL1* mRNA, seen at 1–3 hr (Fig. 2*A*), preceded the increase in the CD11b and CD14 markers, which began at 16–24 hr (Fig. 2*B*). The increase in these cell surface markers coincided with the acquisition of differentiating morphology, which began to be in evidence on about day 1 [at the time of cessation of DNA synthesis (2)] and became fully manifest on days 2 and 3 (see Fig. 2 legend and refs. 1 and 2). In sum, the increase in expression of *MCL1* preceded phenotypic differentiation and the associated cessation of proliferation.

The association of *MCL1* with induction rather than phenotypic differentiation was further evidenced by the finding that expression of *MCL1* decreased after 3 hr (Fig. 2*A*). Expression had declined by >50% by the onset of the increase in the CD11b and CD14 markers, returning to baseline levels in fully differentiated cells [Fig. 1 (day 3) and Fig. 2]. The pattern of expression of this early-induction gene as it related to differentiation was thus analogous to that of the early-response genes, which are expressed before the onset of DNA synthesis and cell proliferation (5). In a further analogy to the early-response genes, the TPA-induced increase in expression of *MCL1* did not require protein synthesis (data not shown).

The *MCL1*-Encoded cDNA and Predicted Protein. The initial cDNA clone from the differential screen (dif8C) represented ≈0.8 kb from the 3' end of *MCL1*. A panel of overlapping *MCL1* cDNA clones was obtained by repeated rescreening. These clones defined a sequence of 3934 bp, in accord with the longest transcript size, 3.8 kb (Fig. 1). The longest open reading frame was preceded by a Kozak translational initiation sequence (20) and an upstream in-frame stop codon (data not shown) and was predicted to encode a protein of 37.3 kDa (350 amino acids; Fig. 3). This size was confirmed by *in vitro* translation (data not shown).

The amino-terminal portion of the MCL1 protein was found to contain several interesting features (Figs. 3 and 4). The extreme amino terminus had characteristics somewhat
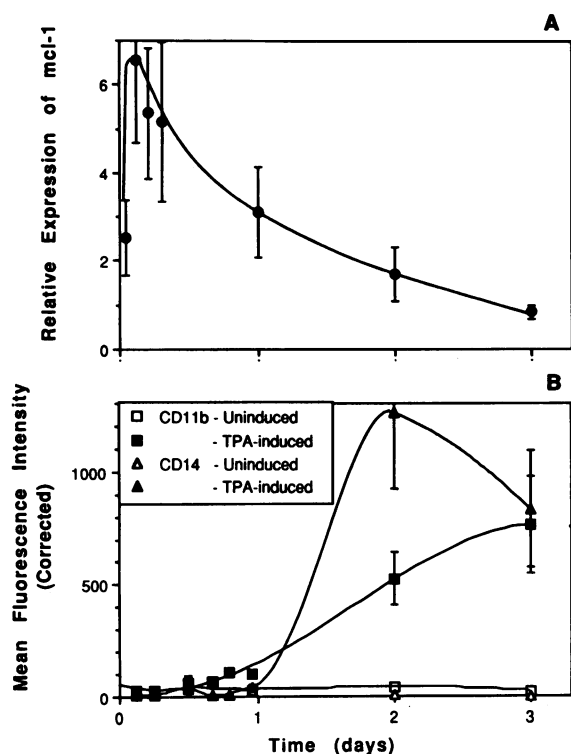
FIG. 2. Relationship between expression of *MCL1* and differentiation in TPA-induced ML-1 cells. (*A*) Time course of expression of *MCL1* (3.8 kb mRNA). (*B*) Time course of appearance of the CD11b and CD14 cell surface markers of differentiation. The percentage of morphologically differentiating cells averaged 40%, 82%, and 90% upon induction with TPA for 1, 2, and 3 days, respectively, and 3.5% in uninduced controls. Immature forms predominated on day 1 (1–3). Cell growth was decreased ≈93% with TPA. Each point represents the mean ± SE of two to five experiments.

reminiscent of a signal sequence (Table 1). Also present were two glycine/alanine-rich regions (overlined in Fig. 3), which surrounded a region enriched in charged amino acid residues. Further downstream were two PEST sequences (21) [enriched in proline, glutamic acid, serine, and threonine (underlined in Fig. 3)], accompanied by four pairs of arginines (asterisks in Fig. 3). PEST sequences and pairs of arginines are present in a variety of oncoproteins and other proteins that undergo rapid turnover (21). Various prominent features of MCL1 are listed in Table 1.


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MFGLKRNAVIGLNLYCGGAGLGAGSGGATRPGGRLLATEKEASARREIGG  50
                                            **
GEAGAVIGGSAGASPPSTLTPDSRRVARPPPIGAEVPDVTATPARLLFFA  100
                      **
PTRRAAPLEEMEAPAADAIMSPEEELDGYEPEPLGKRPAVLPLLELVGES  150
  ***                            **
GNNTSTDGSLPSTPPPAEEEEDELYRQSLEIISRYLREQATGAKDTKPMG  200
                        +
RSGATSRKALETLRRVGDGVQRNHET^V_AFQGMLRKLDIKNEDDVKSLSRVM  250
          ->**
IHVFSDGVTNWGRIVTLISFGAFVAKHLKTINQESCIEPLAESITDVLVR  300
TKRDWLVKQRGWDGFVEFFHVEDLEGGIRNVLLAFAGVAGVGAGLAYLIR  350
                           +                    +
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FIG. 3. Deduced amino acid sequence of MCL1. PEST sequences are underlined and pairs of arginines are marked with asterisks. Areas rich in glycine and alanine (largely neutral) are overlined; these surround a region enriched in charged amino acid residues. The region with sequence similarity to BCL2 is indicated with an arrow, and the hydrophobic carboxyl tail with double lines. Positively charged flanking amino acid residues are marked with plus signs. Amino acid residue 227 was valine in cDNA clones from ML-1 and alanine in those from U-937. Amino acid residue 1 corresponds to nucleotides 61–63 of the cDNA.

It was the carboxyl portion of MCL1 that was found to have significant sequence similarity to BCL2 (Figs. 3 and 4). The carboxyl-terminal 139 amino acid residues of MCL1 and the corresponding portion of BCL2 exhibited 35% amino acid identity and 59% similarity (allowing conservative amino acid substitutions; Fig. 5). Two amino acid stretches within the carboxyl portions exhibited striking similarity (Fig. 4, horizontal striations; Fig. 5, asterisks). Upstream of each of these stretches, a region with the potential to form amphipathic α-helix was found (Fig. 5, alternating single/double underlines). The similarity between BCL2 and the BHRF1 gene product from Epstein–Barr virus is also in the carboxyl portion [≈25% identity (10, 11); Fig. 5, bottom two lines]. Curiously, the few amino acids conserved among the three genes were scattered throughout the carboxyl region (Fig. 5, bold letters). Furthermore, the carboxyl portions of MCL1 and BHRF1 were more closely related to BCL2 than to each other. Overall, distant but highly significant sequence similarity characterized the carboxyl portions of these gene products.

At its extreme carboxyl terminus, the predicted MCL1 contained a potential membrane-spanning domain. This consisted of 20 hydrophobic amino acid residues flanked on either side by a positively charged residue (double lines in Figs. 3 and 5). A similar domain is present in both BCL2α [the major form of BCL2] (7) and BHRF1 (10, 11).

Parallels between *MCL1* and *BCL2* continued downstream of the protein coding region: *MCL1* had a long 3' untranslated region [2.8 kb (Fig. 4)], as does *BCL2* (7–9). This region of *MCL1* had a repeat of cytosine and adenine residues following the protein-coding region [nucleotides 1148–1162 = $(ACC)_5$; # in Fig. 4]; a similar repeat is present in *BCL2* [$(CA)_5GACAGA(CA)_7$; ref. 8]. The 3' untranslated region of *MCL1* contained several potential polyadenylylation sites (AATAAA at nucleotides 1265, 2401, 2501, 3912, and 3929; Fig. 4). This could relate to the two transcripts observed (Fig. 1), since the 2.5-kb transcript was not detected with probes representing regions 3' of the polyadenylylation site at nucleotide 2501. The 3' untranslated region of *MCL1* also contained multiple mRNA destabilization signals [ATTTA at nucleotides 1383, 1489, 1859, 2253, 2267, 3038, and 3073 and (T)TATTTAT at nucleotides 2669, 3284, and 3311; ref. 24]; this could relate to the transience of the increase in the *MCL1* mRNA (Fig. 2A). The 3' untranslated region of *BCL2*, likewise, contains multiple copies of both potential polyadenylylation sites and mRNA destabilization signals (7, 8). Translocations involving *BCL2* frequently occur in the 3' untranslated region, usually within the "major breakpoint region" of about 150 nucleotides (7–9, 25). The 3' untranslated region of *MCL1* contained a stretch with sequence similarity to this major breakpoint region (at nucleotide 1515; ## in Fig. 4). Thus, *MCL1* and *BCL2* demonstrated common features (but no long, continuous sequence similarity) in their 3' untranslated regions, in addition to significant sequence similarity in the carboxyl portions of their protein-coding regions.

DISCUSSION

The work described here was aimed at identifying early-induction genes in a differentiating hematopoietic cell line. Its outcome has been the isolation of *MCL1* and the suggestion of the existence of an *MCL1/BCL2* gene family. In retrospect, it is not surprising that a gene expressed early in myeloid differentiation (*MCL1*) would prove to have sequence similarity to a gene important in lymphoid development (*BCL2*). The similarity between *MCL1* and *BCL2* is in their carboxyl portions, where the cellular *BCL2* gene also has similarity to the viral BHRF1 gene. The similarities among these genes are highly significant, although *MCL1* and
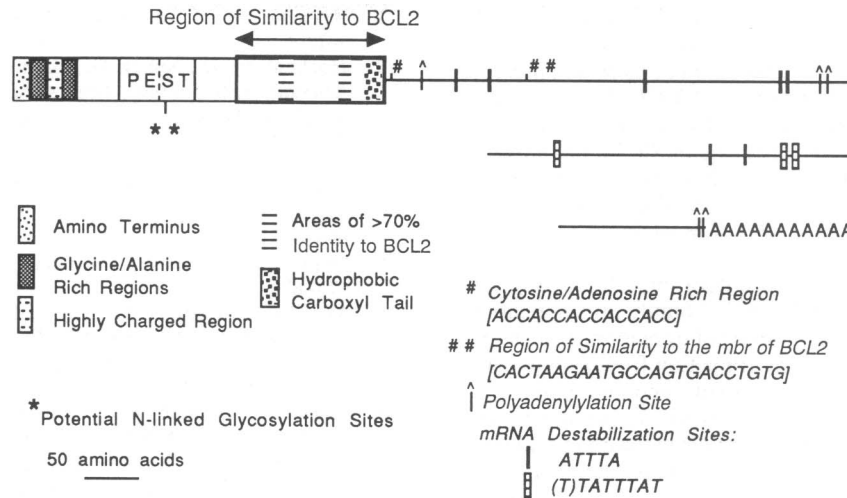
FIG. 4. Schematic representation of the *MCL1* cDNA. The boxed area represents the protein-coding region; this is followed by a line representing the 3' untranslated region. mbr, Major breakpoint region.

BCL2 are clearly not identical [except in two nearly identical stretches (≈7 amino acids each)]. In fact, we probably could not have identified *MCL1* simply by screening at low stringency for genes related to *BCL2*. Paralleling the sequence similarity in the carboxyl regions of the three different genes, the carboxyl portions of the human (7–9) and mouse (22) homologues of *BCL2* exhibit greater conservation than the amino-terminal portions (98% identity in the carboxyl 144 amino acid residues compared to 76% in the remaining amino-terminal portion). Comparable results have recently been obtained in the chicken (23, 26). In sum, *MCL1*, *BCL2*, and BHRF1 may represent a gene family characterized by consistent sequence similarity/conservation in the carboxyl portion.

Each of the three genes in this putative family terminates in a hydrophobic domain with membrane-spanning potential. This hydrophobic region is thought to mediate the membrane association of the *BCL2* protein, which has recently been found in mitochondrial membranes (refs. 12, 27, and 28; but see also ref. 29). BHRF1 is also membrane-associated (10, 11). Thus, the presence of a hydrophobic carboxyl terminus with the potential for membrane association, in addition to sequence similarity in the carboxyl portion overall, appears characteristic of genes in the *MCL1/BCL2* family.

The amino-terminal portion of MCL1 differs from that of BCL2 and BHRF1 in that it contains PEST sequences. This

suggests that the MCL1 protein might be expected to be expressed, like the mRNA, primarily in the early stages of differentiation. BCL2 and BHRF1 do not have strong PEST sequences; however, *BCL2* does demonstrate differentiation-stage-specific expression. For example, the BCL2 protein is present in immature myeloid cells and crypt cells of the intestinal epithelium, its levels declining upon maturation (14, 30–32). It will be interesting to learn whether different representatives of the *MCL1/BCL2* family might exhibit different time-course profiles during hematopoietic cell differentiation.

In addition to characteristic features in their amino acid sequences, the members of the *MCL1/BCL2* gene family exhibit interesting parallels in their origins and patterns of expression. We isolated *MCL1* from ML-1 cells, which derive from a patient who developed acute myeloid leukemia after the remission of a T-cell lymphoma (33); *BCL2* was
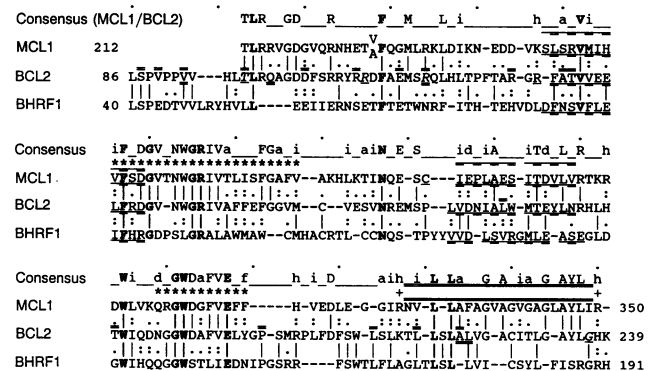
Table 1. Comparison of features present in MCL1, BCL2, and BHRF1

	MCL1	BCL2	BHRF1
Amino terminus with signal sequence-like characteristics	±*	−	+
Regions rich in Gly, Ala, and/or Pro†	+	+	−
PEST sequences	+	−	−
Carboxyl region of sequence similarity	+	+	+
Hydrophobic carboxyl tail	+	+	+

*Amino acid residues 1–27 of MCL1 are positive with SIGSEQ1 and negative with SIGSEQ2.

†Amino acid residues 17–71 of MCL1 consist of 55% Gly + Ala + Pro with neutral residues surrounding charged residues. Analogous regions are seen in human (residues 35–93), mouse (residues 37–90), and chicken (residues 37–87) BCL2, which consist, respectively, of 58%, 46%, and 69% Gly + Ala + Pro. Amino acids 21–42 of BHRF1 consist of 32% Gly + Ala + Pro.

FIG. 5. Alignment of the carboxyl portions of MCL1, BCL2α, and BHRF1. Alignment was performed with the BESTFIT program, gaps being inserted to maximize overlap. Vertical lines indicate amino acid identity; colons indicate amino acid comparison value ≥ 0.5; periods indicate amino acid comparison value ≥ 0.1. Residues that are identical in the three proteins are in bold. The hydrophobic carboxyl tail is shown by double lines flanked by plus signs. The regions of greatest similarity between MCL1 and BCL2 are indicated by asterisks; preceding these are regions with the potential to form amphipathic α-helices (alternating single/double underlines). A consensus sequence for MCL1 and BCL2 is at the top, where similar nonidentical residues were determined by the SIMPLIFY program: a = P, A, G, S, T; i = L, I, V, M; f = F, Y, W; d = Q, N, E, D; h = H, K, R. Single amino acid differences in BCL2 (7–9) are in underlined italics. Single amino acid differences between human (7–9) and mouse (22) BCL2 are doubly underlined and those between human (7–9) and chicken (23) BCL2 are doubly overlined.

identified in patients with follicular B-cell lymphoma (6–9). TPA elicits an early increase in expression of *MCL1* and can combine with other agents to cause similar increases in *BCL2* and BHRF1 (10, 11, 34). Expression of *MCL1* increases early in myeloid cells programmed to differentiate and stop proliferating without dying. Expression of *BCL2* increases in lymphoid cells programmed to remain viable and selected for further differentiation (12–14). Expression of BHRF1 increases early in the lytic cycle of Epstein–Barr virus (it precedes but is not critical for viral replication), and in the serum-induced stimulation of proliferation (10, 11, 35). Expression of genes in the *MCL1/BCL2* family thus appears to be associated with the programming of transitions in cell fate, such as the programming of the transition from proliferation to differentiation and between viability and cell death.

It is not known how these parallels in sequence and expression might translate into parallels in function. Families defined by sequence similarity may have members with similar as well as different functions (e.g., stimulatory vs. inhibitory members of the G-protein family). Thus, *MCL1* could have a function that is similar to, different from, or diametrically opposed to that of *BCL2*. *BCL2* functions in the inhibition of programmed cell death (12, 13); it appears to operate in a variety of cells, such as certain lymphoid, hematopoietic, and other cells (e.g., B memory cells and T cells under specific circumstances) (12, 36–41). *BCL2* is distinct from many oncogenes and growth factor-related genes in that the enhancement of viability may not be accompanied by a stimulation of proliferation, so that viable cells remain in the G_0/G_1 phase of the cell cycle (36–38). The role of deregulation of *BCL2* in tumorigenesis is thought to relate to this enhancement of cell survival, which would allow the accumulation of additional changes (such as rearrangements of the c-*myc* oncogene) (38, 42, 43). One can speculate that *MCL1* might similarly have an influence on cell viability/death in the early stages of induction of differentiation. *MCL1* and *BCL2* are among a growing number of genes known to be expressed in relation to the programming of differentiation/development and viability/death [e.g., the ced-9 gene (44), the fas antigen gene (45), and the adenovirus E1B 19-kDa protein (46)]. One can further speculate that some of these genes may, in conjunction with the various known families of oncogenes and tumor-suppressor genes, play an important role in tumorigenesis and its reversal.

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