Exon Skipping in Mcl-1 Results in a Bcl-2 Homology Domain 3 Only Gene Product That Promotes Cell Death*

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Colin D. Bingle‡§, Ruth W. Craig¶, Brenka M. Swales¶, Vanessa Singleton‡, Ping Zhou¶, and Moira K. B. Whyte‡
From the §Respiratory Cell and Molecular Biology Laboratory, Division of Molecular and Genetic Medicine, The University of Sheffield Medical School, Sheffield, S10 2RX, United Kingdom and the ¶Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755-3835

Mcl-1 is a member of the Bcl-2 family that is regulated transcriptionally and post-transcriptionally, with expression of the full-length Mcl-1-encoded gene product resulting in enhanced cell survival. As reported here, the human Mcl-1 gene can also undergo differential splicing, which yields an internally deleted, death-inducing gene product, Mcl-1ΔTM. Whereas full-length Mcl-1 derives from three coding exons (instead of the two present in Bcl-2 and other anti-apoptotic members of this family), the Mcl-1ΔTM splice variant results from the joining of the first and third exons with skipping of the central exon. Because of the skipped exon and a shift in the reading frame downstream, the Bcl-2 homology domain (BH3) remains intact, whereas the BH1-, BH2-, and transmembrane-encoding domains do not. Mcl-1ΔTM thus has features similar to BH3 only, pro-apoptotic Bcl-2 family members and, accordingly, was found to promote cell death. In addition to a variety of other types of regulation, the Mcl-1 gene appears ideally designed for the generation of either a Bcl-2-like viability promoting or, as reported here, a BH3 only death-inducing gene product.

Apoptosis is a genetically determined cell death program that is critical for the maintenance of tissue homeostasis in the healthy organism. Alterations in apoptosis contribute to the pathological effects seen in a variety of diseases, including cancer and inflammatory disease (1). Apoptosis is controlled by evolutionarily conserved sets of genes with the Bcl-2 family playing a pivotal role (2, 3). The members of this family fall into two groups with opposing functions. Pro-survival family members (e.g. Bcl-2, Bcl-XL, and Mcl-1) inhibit apoptosis, whereas death-inducing members (e.g. Bax, Bid, and Bad) have the opposite effect. The family is characterized by domains of high sequence conservation, termed the Bcl-2 homology (BH)1 domain. Bcl-2 and other pro-survival family members contain BH1, BH2, and BH3 domains, which form a hydrophobic cleft (4) and appear critical for anti-apoptotic function (5). Whereas some death-inducing family members contain all three of these domains (e.g. Bax, Bak, and Bok (6–8)), others contain BH3 only (e.g. Bid, Bad, and Bim). This suggests that BH3 is a minimum requirement for pro-apoptotic function (9, 10), which probably relates to the fact that the exposed BH3 domain can bind to the hydrophobic cleft of pro-survival family members (11). In addition to the BH domains, many family members contain a transmembrane (TM) domain at the C terminus. This anchors the protein to intracellular membranes, such as to the outer surface of mitochondria (12–15). Although this domain may aid in targeting certain family members for function (16, 17), a TM domain is not present in some death-inducing members, namely Bid and Bad (3). In sum, the BH1/BH2/BH3 hydrophobic cleft and the TM anchor appear to contribute to anti-apoptotic activity, whereas the BH3 domain is critical for pro-apoptotic function.

Mcl-1 is a Bcl-2 family member that was originally identified as a gene rapidly up-regulated early in the differentiation of a human myeloid leukemia cell line (ML-1) upon induction with 12-O-tetradecanoylphorbol acetate (PMA) (18). Mcl-1 expression has since been found to exhibit differentiation stage-specific expression in a variety of hematopoietic lineages as well as in epithelial tissues (19, 20). In addition, Mcl-1 can be rapidly induced through anti-apoptotic cytokine-mediated pathways, such as those stimulated by hematopoietic cell colony-stimulating factors and interleukins (21–25). For example, in neutrophils (26), external stimuli that prolong the neutrophil life span (such as lipopolysaccharide and granulocyte-macrophage colony-stimulating factor) cause an increase in Mcl-1 expression (23, 27). An increase in Mcl-1 expression can be induced through a mitogen-activated protein kinase-dependent signal transduction pathway acting on SRF/Elk-1(21, 28), as well as through an Akt/ cAMP-response element-binding protein-regulated pathway (29). In addition to regulation at a transcriptional level, the Mcl-1 mRNA can be rapidly turned over (30). Furthermore, the upstream half of Mcl-1 contains PEST sequences, and the protein is likewise subject to rapid turn-over (15). The downstream half of the protein contains the BH1, BH2, and BH3 domains and terminates in a TM domain (15, 18). Overall, Mcl-1 is a highly regulated gene product and is frequently expressed during particular stages of cell differentiation and/or in response to specific signals.

Increased expression of the endogenous full-length Mcl-1 protein is associated with the maintenance of cell viability, and...
decreased expression with cell death (22, 23, 25, 31). Accordingly, transfection with a construct representing the full-length gene product results in enhanced cell survival, as seen both in the FDC-P1 hematopoietic cell line and in Chinese hamster ovary cells (32–34). Enhancement of cell survival is seen in the presence of a variety of apoptosis-inducing stimuli and at levels of the introduced protein that are readily attainable endogenously. Conversely, loss of the expression of Mcl-1 is associated with cell loss (28, 34, 35). Similarly, in mice that express full-length Mcl-1 as a transgene in hematopoietic tissues (36), lymphoid (B and T) and myeloid cells exhibit enhanced survival, and the life span of mast cells and monocytes is dramatically prolonged. In sum, expression of the full-length BH1/BH2/BH3/TM-containing Mcl-1 gene product results in enhanced cell survival.

Alternative splicing is increasingly being recognized to play a significant role in the regulation of proteins involved in cell death, occurring in a variety of death receptors, Bcl-2 family members, and cell death effectors such as caspases (37). Bcl-x can undergo alternative splicing to yield two forms (Bcl-xL and Bcl-xS) that have opposing biological functions. Bcl-xL and Bcl-xS contain two coding exons separated by a conserved intron in the BH2 domain near the C terminus (38–40). Bcl-xL, which is anti-apoptotic, derives from both of the coding exons in their entirety. Bcl-xS, which is pro-apoptotic, results from the use of an alternate upstream splice donor site that does not lie within the first coding exon (38–40). Other splice forms of Bcl-x also exist, and thus alternative splicing to a variable of different isoforms can occur even with a relatively simple gene structure (37, 41).

We are interested in the role that the Bcl-2 family plays in controlling cell life span in differentiating tissues, such as in the myeloid lineage (both monocytic and granulocytic branches) (42) and in epithelial tissues. Mcl-1 is of particular interest because of its highly regulated pattern of expression in these tissues (20–25). In the research presented here, we describe Mcl-1ΔSTM, a splice variant of Mcl-1 that derives from exon skipping. Although the full-length human Mcl-1 gene product was found to consist of three coding exons (rather than the two coding exons seen in Bcl-2 and Bcl-xL), the Mcl-1ΔSTM variant represented joining of the first and third exons. This does not affect the BH3 domain in the first exon but does affect the BH1, BH2, and TM domains downstream because of the skipped second exon and a change in the reading frame downstream. The features of the Mcl-1ΔSTM-encoded gene product are thus reminiscent of BH3 only pro-apoptotic family members such Bid and Bad. Accordingly, transfection with Mcl-1ΔSTM was found to result in cell death. In addition to regulation at the level of both transcription and turn-over, the Mcl-1 gene appears ideally designed for the generation of either a Bcl-2-like viability promoting or a BH3 only death-inducing gene product.

**MATERIALS AND METHODS**

**Isoleus and Culture of Human Cells and Cell Lines—**Human peripheral blood neutrophils and mononuclear cells were isolated from normal healthy donors by dextran sedimentation and plasma-Percoll gradient centrifugation as described previously (43) and cultured in RPMI and 10% fetal calf serum in Teflon pools. Monocyte-derived macrophages were obtained by purification of peripheral blood mononuclear cells and adherence to tissue culture plasticware in serum-free medium for 1 h followed by culture for 4–5 days in Iscove’s modified Dulbeco’s medium and 10% autologous serum (44). Established cell lines, ML-1, BL41–3, K562, Jurkat, A594, H441, Hep2G, and MCF-7 were cultured in RPMI with 10% fetal calf serum, 10 mM glutamine, and 50 μg/ml streptomycin and penicillin. PMA stimulation of BL41–3 cells was performed using 1–50 ng/ml PMA (20 ng/ml). Total RNA was isolated from peripheral blood cells and cell lines by the RNAeasy system (Qiagen). First strand cDNA was generated by oligo(dT)-primed reverse transcription using avian myeloblastosis virus reverse transcriptase (Promega) and under standard conditions (1 μg of RNA in a total volume of 50 μl). Aliquots (5 μl) of first-strand cDNA were subjected to PCR using sets of primers for Mcl-1-F5, 5′-GGT AGG AGT CGG GTT CTC TTC CCC; Mcl-1-R4, 5′-AAA TTA ATG AAT TCG GGG; Mcl-1-R7, 5′-TCC TCT TGC CAC TTT CCT TTC. Reactions used the following primer combinations F3 and R4, F4 and R4, or F5 and R7. PCR was performed in a PCT2000 thermocycler (MJ Research) for 30 cycles with denaturation at 94°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. Aliquots of the reaction products were subjected to electrophoresis in 1% agarose gels (Tris-acetate-EDTA buffer) and visualized by staining with ethidium bromide. The bands obtained with the F4/R4 and F5/R7 primer combinations were excised and cloned using the TOPO pCR II and bidirectional eukaryotic (pCR3.1 TA cloning systems (Invitrogen). Representative minipreps were chosen for sequence analysis using an ABI 373 automated sequencer. A complete sequence was obtained from multiple clones.

**Cloning, Mapping, and Sequencing of the Human Mcl-1 Genomic Locus—**A 3′-P-labeled probe containing the entire Mcl-1 coding region (p3.2; (18)) was used to screen a human leukocyte genomic in the EMBL vector (CLONTECH catalog no. M11001; Palo Alto, CA), using standard techniques. The resulting positive clones were characterized by restriction enzyme mapping. Selected restriction enzyme fragments were isolated from agarose gels, subcloned into the pBluescript SKII+ vector (Stratagene), and sequenced on both strands.

**Identification of Mcl-1 Transcriptional Start Sites—** Primer extension was carried out using an oligonucleotide primer complementary to the Mcl-1 coding region in its upstream portion (primer #105C primer, 5′-CCCCACATGTAGGTTGGATCGTGACCGC-3′). Counting the ATG of the initiator methionine as the first through third nucleotides, the primer used represents nucleotides 23–52 (or nucleotides 77–106 of the p3.2 Mcl-1 cDNA (18, 21)). After 5′-end-labeling with 32P using T4 polynucleotide kinase, the primer (~2 × 107 cpm) was hybridized to total RNA (20 μg) from ML-1 cells that had been treated with PMA to increase expression of the Mcl-1 mRNA (18, 21). As a control, the primer was hybridized in parallel to yeast tRNA (20 μg). Hybridization was carried out at 30 °C for 14 h in 10 μl Tris-Cl buffer, pH 8.3, containing 150 μM KCl and 1 μM EDTA. Reverse transcription with avian myeloblastosis virus reverse transcriptase (Promega) was carried out at 42 °C for 90 min in 50 μl Tris-Cl buffer, pH 8.3, containing 10 μM MgCl2, 50 mM dithiothreitol, 500 μM each of dATP, dGTP, dCTP, and dTTP (7.5 μM each), and 0.15 μg/μl actinomycin D. After stopping the reaction by the addition of EDTA (to 20 μM), samples were treated with RNase A (Sigma) at 37 °C for 30 min, extracted with phenol/chloroform, precipitated with ethanol, and subjected to gel electrophoresis. The S1 nuclelease protection assay was carried out using a 3′-P-labeled single-stranded DNA probe complementary to Mcl-1 in the upstream portion of the human gene and extending into the 5′-flanking region immediately upstream. This probe was prepared using a template representing single-stranded Mcl-1 genomic DNA derived from an M13 subclone containing the transcript strand of a 567 base SacI-NcoI Mcl-1 genomic subclone from this region (clone SN0.6, which extends from the NorI site within Mcl-1 exon 1 (see Fig. 2) to a SacI site ~0.6 kb upstream). The 3′-P-labeled #105C oligonucleotide primer used above was annealed to this template, and the extension reaction was carried out. The double-stranded DNA product obtained was then cleaved at a DraI site 153 nucleotides upstream from the initiator methionine (18, 21) (see also Fig. 3B). After alkaline-denaturing gel electrophoresis, phenol extraction, and ethanol precipitation, the resultant 5′-end-labeled single-stranded probe, 205 bases in length (2 × 105 CFPM), was hybridized with total RNA (30 μg) from ML-1 cells treated with PMA (18, 21). As a control, the probe was hybridized in parallel to yeast tRNA (20 μg). Hybridization (20 μl reaction) was carried out at 30 °C in 40 μl PIPES, pH 6.4, containing 80% formamide, 400 mM NaCl, and 1 mM EDTA. After 14 h, the hybridization reaction mixture was diluted by the addition of 300 μl of S1 nuclease buffer (50 μM sodium acetate, pH 4.5, containing 0.28 μM NaCl and 4.5 μM ZnSO4) to which denatured calf thymus DNA (10 ng/μl) was added. The sample was added to a decrease (300 units) was carried out at 30 °C for 60 min and was terminated by the addition of 80 μl of stop buffer (4 M ammonium acetate, pH 8.0, containing 20 mM EDTA and 40 μg/ml yeast tRNA). After ethanol precipitation and resuspension in formamide, the protected fragments were assessed by gel electrophoresis on a DNA sequencing gel alongside a sequencing reaction carried out with the 105C primer and

**RT-PCR Detection of Mcl-1ΔSTM in Human Cells and Cell Lines—**
the SN0.6 M13 clone as template. The products of the primer extension reaction (above) were analyzed on the same gel.

RNase Protection Analysis—RNase protection analysis was performed using the multiprobe RNase protection analysis system from Pharmingen. A specific probe set was generated which contained probes to Bcl-xL, Mcl-1 s/D, Mcl-1 s/M, and insertless pEGFP-C1; 0.25 μg of total RNA was hybridized with the hAPO-2 probe set (Pharmingen). The RNase protection analysis reactions were carried out as described in the protocol provided by the manufacturer, using 1–3 μg of total cell RNA and dilutions of the positive control RNAs. The reaction products were resolved on 5% sequencing gels in 0.5× TBE buffer. The RNase protection analysis (above) were analyzed on the same gel.

Western Blotting and in Vitro Translation—For Western blotting, cell extracts were prepared using a bromophenol blue lysis buffer (containing 80 mM Tris/HCl, pH 7.5, 2% SDS, 20% glycerol, 0.001% bromophenol blue, 0.1 mM dithiothreitol, 1 mM leupeptin, and 2 mM phenylmethylsulfonyl fluoride). Electrophoresis (12% SDS-polyacrylamide gel electrophoresis) was carried out with the equivalent of 1–3 × 10⁶ cells being run in each lane, protein molecular weight markers (Amersham Pharmacia Biotech) being run with each gel. Following electrophoresis, samples were blotted onto Trans-blot nitrocellulose membranes (BioRad) using a semi-dry blotter, and protein transfer was confirmed by staining with Ponceau S. A rabbit polyclonal antibody raised against a peptide representing amino acids 121–139 of human Mcl-1 (Santa Cruz, SC-819) was used at a final dilution of 1:200, and detection was with ECL (Amersham Pharmacia Biotech). The specificity of this antibody was confirmed using specific blocking peptides corresponding to the peptide used as the initial immunogen (Santa Cruz, SC-819P). To prepare authentic Mcl-1 proteins as standards for Western blots, in vitro transcription and translation were carried out using the wild-type Mcl-1 and Mcl-1 s/D constructs in TOPO pCR II. A coupled transcription/translation system (Promega) was used according to the manufacturer’s instructions with 2-μl aliquots of the reaction products being loaded onto the Western blots.

Transient Transfection with Mcl-1 s/D Expression Constructs to Assess Effects on Cell Death—Two types of expression constructs were prepared for use in transfection into mammalian cells; in one case Mcl-1 or the Mcl-1 s/D variant were fused to green fluorescent protein and in the other case they were expressed in the pCR-3.1 vector. The Mcl-1 RT-PCR reaction products from the 4F/4R primer pair reactions (cloned in pBluescript, digested with BamHI and EcoRI, and sequenced) were expressed in pCRTM vector and tested for the existence of such an internally deleted Mcl-1 by using primers from either side of the putative deletion in RT-PCR. Two distinct cDNA products were obtained, one of which corresponded to the size expected for full-length Mcl-1 and the other of which was ~250 bp shorter (Fig. 1A). The shorter cDNA products were obtained using three different combinations of primers (F3 and R4, F4 and R5, and F5 and R7). This result was seen using RNA from a variety of different types of cells, including primary hematopoietic cells (e.g., neutrophils) as well as cell lines of both hematopoietic and nonhematopoietic (e.g., epithelial) origin. The smaller of the cDNA products consistently stained less intensely than the longer product, suggesting that it could represent a transcript of lower abundance as is seen with Bcl-xB, which is generally less abundant than Bcl-xL.

Cloning and sequencing of the above cDNA products demonstrated that the shorter product matched the full-length Mcl-1 sequence, whereas the shorter product contained the internal deletion of nucleotides 749–996. This deletion lies within the protein coding sequence, which is encoded by nucleotides 61–110 of the full-length Mcl-1 cDNA (#L08246). Both the shorter and the longer product contained the Mcl-1 start site for translation (18) as well as a stop codon, as seen upon sequencing of the products obtained with primers (F5 and R7) representing regions upstream (F5) and downstream (R7) of the Mcl-1 coding sequence. In sum, the shorter cDNA appeared to represent a variant Mcl-1 containing a 248-bp deletion within the coding sequence.

Except for the deletion, the nucleotide sequence of the variant Mcl-1 matches that of the full-length cDNA; however, the sequence downstream of the deletion is in a different reading frame in the variant. Therefore, the amino acid sequence predicted for the variant is identical to that of full-length Mcl-1 upstream of the deletion but differs at the C terminus. Specifically, the first 229 (out of 271) amino acid residues of the internally deleted variant are identical to full-length Mcl-1, whereas the C-terminal 42 residues are not (Fig. 1B). The deletion thus does not affect the upstream portion of Mcl-1 encoding the PEST sequences and the BH3 domain but does affect the downstream portion encoding the BH1, BH2, and TM domains (Fig. 1, B and C). Not only does the C terminus of the deleted variant differ from full-length Mcl-1, but it also does not match other proteins in the database. Because the internally deleted variant encodes a shorter protein with an altered C terminus, we term it Mcl-1 s/D, where s designates short and ΔTM designates lack of the TM domain. This variant is in some respects similar to Bcl-xL, which retains the BH3 but not the BH1 and BH2 domains. A difference is that the splicing...
that yields Bcl-x, does not affect the reading frame and therefore does not affect the C-terminal TM domain. However, other pro-apoptotic family members, namely Bid and Bad, contain BH3 but not the BH1, BH2, or TM domains. Overall, in addition to the full-length Mcl-1 transcript, a variety of cells contain a variant, Mcl-1_{s\_\text{ATA}}M, which has characteristics reminiscent of...
death-promoting Bcl-2 family members.

The Mcl-1s/TM Variant Derives from Skipping of a Central Coding Exon Delineated by an Intron Downstream of BH3 along with the Conserved Intron in BH2—The splicing that yields Bcl-x, results from the use of an alternate upstream splice donor site lying within the first coding exon (39), and Bcl-x, like other anti-apoptotic family members, contains a single intron within the protein-coding region (near the C terminus) (38–40, 45, 46). An intron at this position is a highly conserved feature of the Bcl-2 family (37, 47–51). For clues as to how the Mcl-1s/TM variant arises, we characterized ~30 kb of the Mcl-1 human genomic locus. In contrast to other anti-apoptotic family members, Mcl-1 was found to consist of three coding exons (Fig. 2A). Thus, Mcl-1 contains an intron just downstream of BH3, in addition to the conserved intron further downstream in BH2 (Table 1). Exon 1 is G/C-rich and encodes the first 229 amino acid residues containing the PEST sequences and BH3; exon 2 encodes BH1 and a portion of BH2, and exon 3 encodes the remainder of BH2 and the C-terminal TM domain. The Mcl-1s/TM variant represents exon 1 joined to exon 3 with skipping of exon 2 (Fig. 2C). Exons 2 and 3 are not in the same phase (Table 1), which accounts for the shift in reading frame at the C terminus. Whereas Bcl-2 and other anti-apoptotic family members do not contain an intron comparable to a Mcl-1 intron 1, an intron is present at this location in pro-apoptotic Bax (Bax intron 3) (8).

Mcl-1 Transcriptional Initiation Sites Lie Directly Upstream of the First Coding Exon without an Intervening 5′-Untranslated Exon—Both Bcl-2 and Bcl-x contain upstream untranslated exons in addition to the two coding exons (38, 40). To look for such upstream exons and to identify the start site(s) of transcription in Mcl-1, we carried out primer extension analysis and S1 nuclease mapping. In primer extension, the most abundant of the products obtained extended to about 70 bp upstream of the Mcl-1 translation start site (Fig. 3A, lane 3); additional products extended 10 bp further upstream (these two positions are indicated with arrows to the left of the sequencing ladder in Fig. 3A). Transcription thus appeared to initiate in the region of two tandem initiator sequences (52) present at this location in the immediate 5′-flank of Mcl-1 (Fig. 3A left column of letters and Fig. 3B, double underlines). The primer extension analysis did not specify initiation exclusively at a single nucleotide within the region containing these tandem sequences. Instead, initiation appears to occur at at least two major sites within an ~10-bp stretch, possibly reflecting the presence of two initiator sequences. S1 nuclease mapping also yielded a protected fragment indicative of transcriptional initiation in the region of the initiator sequences (Fig. 3, lane 2). The longest fragment obtained upon S1 nuclease mapping corresponded most closely to the more downstream of the sites indicated by primer extension. Additional shorter fragments were also seen, which could reflect incomplete protection from S1 nuclease digestion. The reason that the more upstream site was not detected by S1 nuclease mapping could relate to the fact that it was associated with less abundant primer extension products. It could also relate to the presence of a repeated sequence in the tandem initiators (i.e. CACTTC), which could allow the formation of a loop upon binding to the probe. Whatever the case, both mapping methods suggested that a majority of Mcl-1 transcripts initiated from the more downstream of the two initiator sequences; this location was therefore designated as position +1 (indicated with an asterisk in Fig. 3).

Fig. 3B shows the position of the initiator sequences relative to other features of the immediate 5′-flank of Mcl-1. Upstream from the initiator sequences lie the Ets and SRF binding elements previously found to have a role in the control of tran-

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**Fig. 2.** The Mcl-1 human genomic locus contains an intron downstream of BH3, in addition to the conserved intron further downstream in BH2. A, intron/exon structure and GC content of the human Mcl-1 genomic locus. A ~8-kb region of human genomic DNA that included the Mcl-1 gene was sequenced, using genomic subclones as shown in B. The three Mcl-1 coding exons are shaded gray, with the 3′-untranslated region being white. The two introns are also white, and the 5′- and 3′-flanking regions are hatched. The regions encoding the BH1, BH2, BH3, and TM domains are indicated, where BH2 is encoded in exons 2 and 3. A GGCCC repeat and two initiator sequences that lie upstream of exon 1 are indicated (black dot and upward pointing arrow; see also Fig. 3). Also indicated are two polyadenylation signals in the long 3′-untranslated region; these correspond to two transcripts previously reported (3.8 and 2.5 kb (18)), the former representing the full-length cDNA and the latter polyadenylation at nucleotide 2425 of the cDNA sequence (see accession no. AF118124). Alu sequences are present in the 5′- and 3′-flanking regions of Mcl-1 and embedded within the upstream Alu sequence is a retrovirus long terminal repeat with the properties of a 3′-long terminal repeat; these sequences are shown as heavily hatched regions. The C+G and CpG content is diagrammed below the sequence. The complete genomic sequence has the accession number AF198614. B, two lambda phage clones that span the Mcl-1 genomic locus (B3 and B5 in EMBL-3) were subcloned and used for restriction enzyme mapping and sequencing. Restriction enzyme digestion sites are indicated at the top, as follows: B, BamHI; E, EcoRI; N, NotI, X, XhoI; Xb, XbaI, S, SalI (the latter from the EMBL-3 vector). The region represented in A is the region between the Xb1 and Xb2 sites, as indicated. Selected genomic subclones (in pBluescript) are shown at the bottom, where these are designated according to the restriction enzyme sites at their 5′- and 3′-ends (abbreviated as above except that X designates XhoI and K designates KpnI and where the numbers indicate the subclone size (in kb)). C, the Mcl-1s/TM variant represents juxtaposition of exon 1 to exon 3 with the skipping of exon 2, as compared with full-length Mcl-1 in which exons 1, 2, and 3 are represented.
The positions of Mcl-1 introns 1 and 2 are shown. Intron 1 lies just downstream of the BH3 domain and intron 2 lies within the BH2 domain (see also Fig. 2: A terminal intron in BH2 is a highly conserved feature of the Bel-2 family. An intron at a position similar to that of Mcl-1 intron 1 is present in the Bax gene (intron 2), but not in Bel-2. The total number of nucleotides in Mcl-1 introns 1 and 2 is indicated in parentheses. Dashes were inserted to optimize sequence alignment (4). Subscripts indicate the numbering of the full-length Mcl-1 cDNA and amino acid sequence (the latter in bold).

| Consensus | Splice donor | Splice acceptor |
|-----------|--------------|-----------------|
| Mcl-1     | AC CAC GAG ACG GTC TTC CAA G746 | GC ATG CTT CCG AAA CTG GAC |
| Intron 1  | N H E T V F Q029 | G230_M L R K L D |
| Mcl-1     | CTA GTI AAA CAA AGA GCC TGG9996 | GAT GGG TT TTG GAG GAT TT |
| Terminal intron (intron 2) | L V K Q R G W312 | D313 G F V E F |
| Bcl-2     | ATC CAG GAT AAC GGA GCC TGG | GAT GCC TTG GAA CTG |
| Terminal Intron | I Q D N G G W | D A F V E L |
| Bax       | ATG GAG CTG CAG AG | GAT ATT GCC GCC GTG GAC |
| Intron 3  | N R E - L Q | R M I A V D |
| Bax       | ATC CAA GAC GAG GGT GGT TGG | GAG GCC CTC TCC TCC TAC |
| Terminal intron (intron 5) | I Q D N G G W | D G L L S Y |

The introduced fusion protein are identified based on green fluorescence and can then be examined for the morphologic features of cell death (53, 54). We found that A549 airway epithelial cells expressing pEGFP-C1-Mcl-1watM as detected by green fluorescence, exhibited cell shrinkage (Fig. 4B) as well as nuclear condensation (Fig. 4C) and blebbing (Fig. 4D) as nuclear condensation (Fig. 4C) and blebbing (Fig. 4D), hallmark of cell death by apoptosis (53, 54). In contrast, cells expressing the pEGFP-C1 vector or full-length pEGFP-C1-Mcl-1 (Fig. 4A and legend) remained morphologically normal and viable, as expected based on previous experiments (32–34).

To confirm the above observations, we used the pCR3.1-Mcl-1watM construct that expresses Mcl-1watM itself rather than a fusion protein. A549 cells transfected with this construct (but not mock-transfected controls) expressed the Mcl-1watM-encoded protein, detected by anti-Mcl-1 antibodies as a protein corresponding in size to authentic in vitro translated Mcl-1watM (Fig. 4E, lane 4). Endogenous full-length Mcl-1 was also detectable in these cells, but its level of expression was not altered in the presence of Mcl-1watM. Although the level of expression of Mcl-1watM appears lower, the relative levels of the variant and full-length Mcl-1 cannot be determined as the former is present only in transfected cells, whereas the latter represents in the entire cell population. Whatever the relative levels, it was clear that the introduced Mcl-1watM protein was expressed in this system. We therefore performed co-transfection experiments with pCR3.1-Mcl-1watM, along with pCMV-β-galactosidase as a marker for transfected cells, to test the effect of expression on cell death. Transfected (i.e. β-galactosidase expressing) cells were found to undergo cell death exactly as described above with pEGFP-C1-Mcl-1watM, an effect that was not seen with the insertless pCR3.1 vector or full-length pCR3.1-Mcl-1 (results not shown). An identical effect was seen in another epithelial cell line, HeLa. Upon transfection with pCR3.1-Mcl-1watM, HeLa cells expressing the β-galactosidase marker rounded up and exhibited shrinkage/blebbing (Fig. 4G), as did cells transfected with a Bax expression vector. In contrast, in parallel cultures transfected with the insertless vector or full-length pCR3.1-Mcl-1 (Fig. 4F and legend), the majority of cells retained normal morphology and viable. Cells expressing β-galactosidase were scored as having either a normal, viable morphology (flat, healthy cells) or the altered morphology characteristic of cells dying by apoptosis (dense, rounded, detached cells). This analysis showed that expression of the Mcl-1watM isoform is nearly as effective as Bax in inducing cell death (Fig. 4H). In two different cell types, in sum,
FIG. 3. Mcl-1 transcription initiates adjacent to tandem initiator sequences directly upstream of the first coding exon. A, Mcl-1 transcriptional start sites were mapped using primer extension analysis (products in lane 3, yeast tRNA control in lane 4) and S1 nuclease digestion (protected fragments in lane 2, yeast tRNA control in lane 1, probe in lane 5). The sequencing ladder (lanes A, T, G, and C) was generated using the #105C primer employed in the mapping (see "Materials and Methods"). The RNA was from PMA-treated M1-1 human myeloid leukemia cells, the cell line from which Mcl-1 was originally cloned (18). Arrows to the left of the sequencing ladder indicate the two major positions where primer extension products were seen (120 and 130 bp upstream from the 5' end of the #105C primer). The more abundant primer extension products were found at the position of the lower arrow, as opposed to the upper arrow 10 bp upstream. The lower position was thus designated +1. This position is indicated with an asterisk on the Mcl-1 coding sequence, which is listed in the column at the left. The sequence as listed includes the two tandem initiator sequences at 69–82 bp upstream of the start site of Mcl-1 translation (see B below). The sequence of these initiators is CCACCTCCTACTTC, where the consensus for initiator sequences is YYA X (T/A) YY. The sequence in the right column is that of the complementary strand, which was that sequenced. The distance (bp) indicated at the right side of the gel represents the distance from 5'9-end of the #105C primer. B, the sequence of the immediate 5'-flank of the human Mcl-1 gene is shown in comparison to the comparable region of the mouse Mcl-1 5'-flank. The tandem initiator sequences located 69–82 bp upstream of the start site of translation of the human gene are double underlined, where a similar sequence is present in the mouse sequence. The site designated as +1 in human Mcl-1 (see A and under "Results") is in bold and is indicated with an asterisk. The +1 site for mouse Mcl-1 is similarly indicated. The tandem initiator sequences lie in break between an upstream CpG-rich area (labeled and indicated with an arrow) and the G/C-rich first exon downstream (see Fig. 2A). The GGCCCC repeat region is dot underlined and CpG pairs are in italics. The numbering in bold (in parentheses) indicates the position relative to the +1 site. The numbering that is not in bold refers to the numbering of the 8253 kb of sequenced human genomic Mcl-1 (diagrammed in Fig. 2A) or the corresponding sequenced area of the mouse Mcl-1 gene (25, 29).
transfection with Mcl-1$_{\Delta\text{TM}}$ resulted in cell death.

Endogenous Mcl-1$_{\Delta\text{TM}}$ Is Expressed at Lower Levels than Full-length Mcl-1 in Viable Cells—The initial RT-PCR assays above suggested that Mcl-1$_{\Delta\text{TM}}$ was expressed at lower abundance than full-length Mcl-1. To confirm this, we used an RNase protection assay in which the probe contains sequence from exons 2 and 3 and thus protects fragments of different sizes for Mcl-1$_{\Delta\text{TM}}$ and full-length Mcl-1. We found the pro-
Mcl-1 is emerging as a highly regulated member of the Bcl-2 family, and the results reported here demonstrate an additional, heretofore unrecognized form of regulation, alternative splicing. The mechanism of generation of the novel Mcl-1<sub>s/TM</sub> isoform involves exon skipping and thus differs from that seen in the case of Bcl-x<sub>S</sub>. This mechanism depends upon the presence of three coding exons in Mcl-1 instead of the two coding exons seen in other anti-apoptotic members of this family. These three coding exons arise because of the presence of an additional intron just downstream of the BH3 domain of Mcl-1, along with the conserved intron in BH2 near the C terminus. The elimination of the central second exon along with a shift in reading frame downstream results in the elimination of the BH1, BH2, and TM-encoding domains. In accordance with the resemblance to BH3 only pro-death family members, exogenous introduction of the Mcl-1<sub>s/TM</sub> variant was found to induce cell death. Similarly, populations of cells that were largely healthy and viable exhibited low levels of Mcl-1<sub>s/TM</sub> as compared with full-length wild-type Mcl-1.

Other members of the Bcl-2 family can also undergo differential splicing (37, 39, 41, 49, 57–59). In terms of anti-apoptotic family members, the splicing of Bcl-x<sub>L</sub> to Bcl-x<sub>S</sub> involves the use of an alternate splice site within the coding sequence (39). Bcl-w can also undergo alternate splicing, and in this case splicing is to an adjacent gene (45). With both Bcl-x and Bcl-2, unspliced transcripts that read through into the intron have also been reported (37). The mechanism involved in the splicing of Mcl-1 differs from that seen with these other anti-apoptotic family members in that it involves exon skipping, where the subsequent exon is placed in an altered reading frame. It is the presence of an additional intron in Mcl-1 downstream of BH3, along with the conserved intron further downstream in BH2,
that allows for this ability to skip an exon and eliminate BH domains critical for anti-apoptotic effects.

Although two coding exons are present in anti-apoptotic family members so far characterized (other than Mcl-1) (38, 40, 45, 46), multiple coding exons (5–6) are present in pro-apoptotic family members. This is because of the presence of 3–5 introns in the coding region (37–47–51), where the terminal intron is that which is conserved throughout the family. For example, additional introns in the Bax gene place BH1, BH2, and BH3 on separate exons. A variety of splice variants have been identified; this includes Bax8, where exon 4 is spliced to exon 2 (in frame) with skipping of exon 3, as well as BaxY, where exon 3 is spliced to exon 1 (with a change in reading frame) with skipping of exon 2 (8, 37). The activity of these various variants has not been completely elucidated. Differential splicing is also seen in other pro-apoptotic family members; in Bok/Mtd, exon 2 can be skipped (49) and, in Bim, alternate splicing can alter the N terminus of the encoded gene product (10). Although the altered splicing of Bok results in retention of a similar function, the different variants of Bim exhibit differences in pro-apoptotic efficacy. One of the introns in pro-apoptotic Bax lies downstream of BH3 in a position analogous to the additional (first) intron in Mcl-1 (8). Other pro-apoptotic family members, namely Bid and Bak, also contain an intron downstream of BH3 (47, 48, 51). However, in the latter two genes these introns are not at positions identical to that of intron 1 in Mcl-1 and the analogous intron in Bax. The alternate upstream splice site within the Bcl-x first coding exon that is used in the production of Bcl-xL, likewise lies downstream of BH3, although this site is again not located at a position identical to that of the intron in Mcl-1 (39). Overall, the multiple introns in pro-apoptotic family members provide a variety of possibilities in terms of differential splicing. Moreover whereas differential splicing through exon skipping, as seen here with Mcl-1, has not been reported for other anti-apoptotic family members, it is common among pro-apoptotic members.

Whereas these studies demonstrate that the human Mcl-1 gene can undergo differential splicing into two distinct isoforms, it remains to be determined how these isoforms function and are coordinated in vivo. It has been suggested that anti-apoptotic members of the Bcl-2 family prevent cell death by inhibiting the action of adaptor proteins involved in the initiation of the caspase cascade, although this is controversial (60). Pro-apoptotic Bcl-2 proteins may then bind to anti-apoptotic family members and neutralize the block on the adaptor proteins to allow the death cascade to develop. The pro-apoptotic activity of several BH3 only Bcl-2 family members has been shown to be regulated in a post-translational manner by protein–protein interactions, which make these proteins unavailable to initiate the death pathway. For example Bad is bound in a phosphorylation-dependent manner to 14–3–3 proteins, which sequesters the protein in a form that is unable to associate with Bcl-2 and Bcl-xL (61). Upon dephosphorylation, Bad translocates to bind Bcl-2 and Bcl-xL and induces the cell death cascade. Bim, another BH3 only protein, is sequestered in the cytoskeletal associated motor complex bound to LC8, a cytoskeletal protein-protein interactions, which make these proteins unavailable to initiate the death pathway. For example Bad is bound in a phosphorylation-dependent manner to 14–3–3 proteins, which sequesters the protein in a form that is unable to associate with Bcl-2 and Bcl-xL (61). Upon dephosphorylation, Bad translocates to bind Bcl-2 and Bcl-xL and induces the cell death cascade. Bim, another BH3 only protein, is sequestered in the cytoskeletal associated motor complex bound to LC8, a cytoskeletal protein.
