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Research Article

Functional analysis of the human *MCL-1* gene

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Abstract. We have isolated a 6.5-kb human genomic fragment that encodes the *MCL-1* gene. Comparison of the coding region with the published full-length cDNA reveals that the gene contains three exons and two introns, and that our clone contains 370 bp of the 3′-untranslated region. We have mapped a major transcriptional start site to 80 residues upstream of the translation initiation codon. Reporter gene assays indicate that regulatory sequences responsible for phorbol ester (PMA)-stimulated activity and granulocyte-macrophage-colony-stimulating factor (GM-CSF)-stimulated activity were located within the first 294 bp of the 5′-flanking region upstream from the transcription start site. A deletion mutant was generated that lacked 47 bp between residues − 215 and − 168: in this mutant, six out of seven GGCCCC repeats and two GCTCA repeats were deleted. Serum-stimulated and GM-CSF-stimulated reporter activity were greatly decreased in this deletion mutant and PMA-stimulated activity was slightly decreased. While the coding and 3′-untranslated regions of the human and mouse genes have significant sequence similarity, there was very little sequence similarity in the 5′-flanking regions of the genes from these two species. Nevertheless, some consensus sequences for a number of transcription-factor-binding sites were detected in the two genes, indicating that transcription may be regulated by similar signalling pathways in these different species.

Key words. Apoptosis; Bcl-2 family; neutrophil; transcription; U-937; promoter; luciferase.

Introduction

The Bcl-2 family of proteins plays a key role in regulating the survival and apoptosis of a variety of cells and tissues [1, 2]. Some of these family members (typified by Bcl-2) protect against cell death, while others (typified by Bax) promote cell death by apoptosis. *MCL-1*, originally cloned as an early induction gene during the differentiation of the myeloid cell line, ML-1 [3], is located on human chromosome 1 [4] and protects against cell death in a variety of experimental systems [5–7]. While Mcl-1 and Bcl-2 may share the ability to promote cell survival, evidence is emerging that the expression of these proteins is independently regulated [8–10] and the proteins may even be found within different subcellular compartments [11]. Current ideas suggest that *MCL-1* expression may be induced prior to *BCL-2* expression during the differentiation of certain cells, and this mediates rapid (albeit short-term) protection against cell death. We have recently shown that mature blood neutrophils express neither Bcl-2 nor Bcl-X, but express Bax constitutively [12]. Survival of these cells appears to be closely related to levels of Mcl-1: levels of this protein are cytokine regulated and correlate closely with rescue from apoptosis following exposure to cytokines such as granulocyte-macrophage-colony-stimulating-factor (GM-CSF) and interleukin (IL)-1β [12].

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In view of the importance of Mcl-1 in regulating cell survival during differentiation of myeloid cells, and also in promoting the survival of human neutrophils, the molecular processes that control its expression need to be identified. The protein has a very short half-life (approximately 30 min in mature neutrophils; unpublished observations) due to the presence of PEST sequences and Arg:Arg motifs that target the protein for cellular proteolysis [3]. Expression is also rapidly induced and so the protein can be subject to acute regulation under appropriate circumstances. In view of its very rapid turnover, cellular levels are maintained essentially by activated transcription. Recently, some information on the properties of the 5′-flanking regions that regulate transcription of the human and murine *MCL-1* genes were reported [13–15]. The coding regions of the human and murine genes are very similar (approx. 70% identity) but the corresponding promoter regions have not been compared.

In this paper we report the characterisation of a 6.5-kb human genomic clone that encompasses the entire intron/exon boundaries of the coding region and some 3.9 kb of the 5′-flanking sequence. We have mapped the transcriptional initiation site and identified both phorbol ester (PMA)- and GM-CSF-stimulated activity of promoter constructs in luciferase reporter gene assays. Remarkably, despite the sequence similarities of the human and murine coding and 3′-untranslated regions, the 5′-flanking regions of the gene have very little overall similarity.

Materials and methods

Reagents. RPMI 1640 was from ICN Biomedicals, fetal calf serum (FCS) from Gibco BRL and PMA from Sigma. rhGM-CSF was a gift from Glaxo. The Dual Luciferase Assay System, pGL-3 Basic reporter vector and pRL-SV40 (R*luc*) were from Promega. A human chromosome 1 genomic library was supplied by UK HGMP Resource Centre (Hinxton, Cambridge, UK) and supplied on gridded membranes. The Random DNA Labelling Kit was from Amersham Pharmacia Biotech and Zeta-Probe GT Genomic Tested Blotting Membrane was from Bio-Rad. All other enzymes used were from Boehringer Mannheim.

Cell lines. The U-937 human macrophage cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and cultured at 37 °C under 5% CO_2 in RPMI 1640 supplemented with 10% bovine calf serum. Cells were fed 16–20 h prior to transfection.

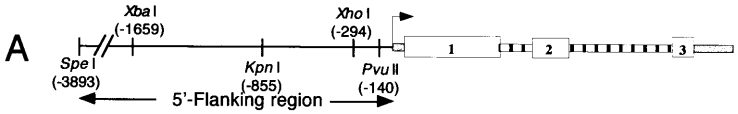
Isolation, cloning and sequencing of human *MCL-1* genomic fragment. A human chromosome 1 genomic library was screened with a 200-bp fragment generated using a Clontech PromoterFinder DNA walking kit and an oligonucleotide designed from the published 5′ terminal region of the human *MCL-1* cDNA (GeneBank accession number: L08246). The sequence of this fragment was subsequently shown to span residues −162 to +39 inclusive. Positive cosmid clones isolated by this screening were identified and obtained from HGMP Resource Centre. Cosmid DNAs were digested using *Eco*RI and *Hin*dIII, separated on a 1% agarose gel which was then Southern blotted and probed with the same probe. A 6.5-kb fragment resulting from *Eco*RI digestion was identified and cloned into pBluescript KS (Stratagene), restriction mapped and sequenced using ABI 373 and Li-Cor Automated Sequencing Systems.

Mapping of the transcription initiation site. Primer extension analysis was used to map the transcription initiation site of the *MCL-1* gene. Twenty micrograms of total RNA from PMA-stimulated U-937 cells was suspended in 20 µl of hybridisation buffer [12] and incubated with 5′-end-labelled oligonucleotide (5′-GGCTGAGAAAACTGGGGAAGAC-3′) at 55 °C for 4 h. Hybridised samples were ethanol-precipitated and resuspended in reverse transcriptase buffer. Reverse transcription was allowed to proceed for 2 h at 42 °C. The products of the primer extension reaction were analysed on a 10% polyacrylamide sequencing gel.

Preparation of reporter gene constructs. An antisense PCR primer (5′-ATTATAAGCTTCTGGAAGGAAGCGGAAGT-3′) was designed to create a *Hin*dIII site in the 5′-untranslated region of the gene and was used with a sense PCR primer (5′-CTAAGACTGTTGTTCCCA-3′) to amplify proximal promoter sequences to include the *Xho*I site (at −294). This PCR fragment was digested by *Hin*dIII and *Xho*I, and directionally cloned into the pGL-3 Basic vector: sequence analysis revealed that a 47-bp GC-rich region between −215 and −168 was missing. This deletion mutant was used as one of the promoter constructs. The same region between *Hin*dIII and *Xho*I sites was completely amplified using the same two primers but in a PCR reaction performed in the presence of 4% (v/v) formamide, and subsequently cloned into the pGL-3 Basic vector, as above. Successful amplification of this fragment was assessed by sequencing. A 3.6-kb promoter region fragment excised from the 6.5-kb original clone using *Spe*I and *Xho*I was ligated to the *Hin*dIII/*Xho*I construct using *Xho*I and *Nhe*I restriction sites in pGL-3 Basic so that the entire 5′-flanking region was cloned. Other reporter gene constructs were created by deletion of upstream promoter regions using restriction enzymes, as indicated in figure 1.

Transient transfection of reporter gene constructs. Cells were transfected as described [15]. They were resuspended in RPMI 1640 supplemented with 10% FCS at a concentration of 1×10^7 in 800 µl of RPMI 1640 to which at least 10 µg of each promoter construct was

added. In addition, 2 μg of pRL-SV40 (luciferase driven by SV40 early enhancer/promoter) was added to each transfection as an internal control. Cells were electroporated with a single pulse from a Bio-Rad Gene Pulser apparatus with a capacitance extender unit (260 V, 960 μFd). Transfected cells were incubated on ice for at least 10 min before and after transfection and then resuspended in 12 ml of RPMI 1640 supplemented with



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                Xba I
AAGGGCTTTA CAAACCTCTA GAGTCAAATG TGCCTTATTA TCAGTACAAA AATAAATGGT GTCAGCTGGG TGCAGTGACT CACACCTGTA ATCCCAGCAC TTTAAGAGGC TGAGGCAGGT -1561

GGATCACCTG AGGCCAGGAG TTTGAGACCA GCCTGGCCAA CATGGTGAAA CCACATTGTC AGGCCTCTGA GCCCAAGCCA AGCCATCGCA TCCCCTGTGA CTTGCACGTA TACATCCAGA -1441

TGGCCTGAAG TAACTGAAGA TCCACAAAAG AAGTAAAAAT AGCCTTAACT GATGACATTC CACCATTGTG ATTTGTTTCT GCCCCACCCG AACTGATCAA TGTACTTTGT AATCTCCCCC -1321

ACCCTTAAGA AGGTTCTTTG TAATTCTCCC CACCCTTGAG AATGTACTTT GTGAGATCCA CCCCTGCCCA CAAAACATTG CTCTCAACTT CACCACCTAT CCCAAAACCT GTAAGAACTA -1201

ATGATAATCC ATCACCCTTT GCTGACTCTC TTTTCGGACT CAGCCCGCCT GCACCCAGGT GAAATAAACA GCCATGTTGC TCACACAAAG CCTGTTTGGT GGTGTCTTCA CACAGACGCG -1081

CATGAAACAC ATCTCTACTA AAAATACAAT AATCAGCTGG GCGAGGTGGC TCACAGCTGT AATCTCAGCA CTTTGGGAGG CCGAGACAGG CAGGTCACTT GAGGCCATGA GTTCGAGACC  -961
                                                                                                                  Kpn I
AGCCTGGCCA ACATCGTGAA AACCCCATCT CTACCAAAAA TACAAAAACT AGCCAGATGT GGTGGCGCAC GCCTGTAATC CCAGCTACTC GGGAGGCTGA GGTACCGAAT CGTCTGAACG  -841

TGGGAAGTGG AGCTTGTAGT GAGCCGAGAT CGCCCCACTG CACTCCAGCC TGGGCAACAG AGCTAGACTG TCTCAAAACA AACAAAAAAT GGTGTCAAGA CTCTCAGACG AGATTCTAAT  -721

GGATTAAGGC CTATATGTAA ATAGCACCAA AGACTATGGA ACAGAGATGG GAGAAGCAAG CAGGGAGGCA GGAATAGTTT AGCTGTGGCA GTTTTAGCTT AGTCCACTTA CATAAATGGT  -601

TCTTTAGGGT AGCACGTGGA GCATCCTCAT TTCCAAACAT TGGACTGAGA GTAGAGAGCT GTGCAAAATA ACCACAAGTC CCCAACTATG CCCTCTTAAT TATCCCTATC ATCTAAGACT  -481

GTTGTTCCCA TCCATCACTG AACTTCCCCG TCCTCTTCCT TCAACCCCTG TGTTAGTCAA TGGTTGAAAT TTTGATTTGG TAAAAAACCT CTGGCGAAAA CCAGCAAAAA GGGCTCACAA  -361
                                                                  Xho I
ATCAGGTCTC AGGGAAGCAC AGAGGTAGCC ACGAGAAGGC CCGAGGTGCT CATGGAAAGA GCTCGAGCCC AGGAGCTCTG GGAGGACCCC AGGCGCTCGG AGCCGCCGTT ACGTAACCGG  -241
                                                                                                                        CRE-BP
                                                                                                                  Pvu II
CACTCAGAGC CTCCGAAGAC CGGAAGGCCC CGCTCAGGCC CCGGCTCAGG CCCCGGCCCC GGCCCCGGCC CCGGCCCCGC CCCGGCCCGG CCGGGCAGCT GGTAGGTGCC GTGCGCAACC  -121
                    Ets   NF-κB                                                                  Sp1
CTCCGGAAGC TGCCGCCCCT TTCCCCTTTT ATGGGAATAC TTTTTTTAAA AAAAAAGAGT TCGCTGGCGC CACCCCGTAG GACTGGCCGC CCTAAAACCG TGATAAAGGA GCTGCTCGCC    -1
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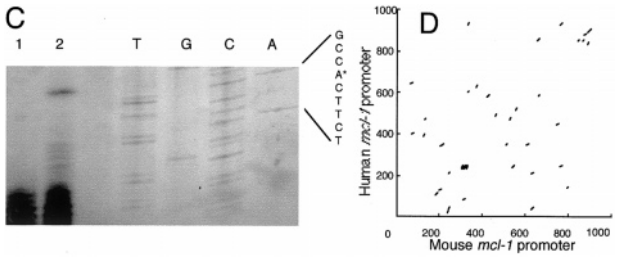

Figure 1. The human *MCL-1* gene structure: transcription start site analysis. (*A*) Structure of the 6.5-kb *Eco*RI fragment isolated from a human chromosome 1 genomic library. 1, 2 and 3 represent the three exons. (*B*) The 1800-bp of the proximal promoter sequence with numbering beginning at the transcription start site (the entire sequence was deposited in GenBank, accession number: AF147742), as mapped in (*C*). Restriction sites used in the generation of reporter constructs are shown in over bars, while potential transcription-factor-binding sites are shown as underbars. Sequences shown in italics denote those deleted in a *Hin*dIII/*Xho*I mutant shown in figure 3. (*D*) Sequence comparison of the first 1000 bp of 5′-flanking sequence of the human gene shown in (*B*), with that of the first 1000 bp of the 5′-flanking region of the mouse gene, taken from Chao et al. [13].

10% bovine calf serum. This was divided into 3×4 ml aliquots and each 4 ml was cultured in the presence of medium alone, GM-CSF (100 U/ml) or PMA (10 ng/ml) in six-well plates. Cell viabilities immediately after electroporation were between 55–65%.

Luciferase assay. Luciferase activity was assayed (as described in the manufacturer's instructions). Transfected cells were harvested after 24 h incubation and cells were lysed in 100 µl of passive lysis buffer provided with the kit; cell debris was pelleted by centrifugation. Twenty microlitres of cell lysate was used for each luciferase assay using a Berthold luminometer. Peak values (which are proportional to luciferase concentration) were recorded. Transfection efficiencies were normalised to *Renilla* luciferase activity. All values were converted to fold luciferase activities by comparison with control plasmid activity (pGL-3 Basic). The half-life of the luciferase protein is approximately 4–5 h [16] and after 24 h incubation of transfected cells, luciferase activity was found to be reproducibly measured in these reporter assays. All experiments were performed in triplicate and data shown represents mean values with error bars showing standard deviations.

Southern and Northern blotting. For Southern blotting, restriction digests were electrophoresed on a 1% agarose gel and DNA was transferred to ZetaProbe membrane by capillary blotting. For Northern blotting, 20 µg of total RNA was separated on agarose/formaldehyde gels and blotted as above. Filters were fixed by baking at 80 °C for 30 min. For Northern blots, membranes were hybridised overnight with a ^{32}P-random-labelled probe which corresponds to a fragment of *MCL-1* mRNA (1236–2350) in a hybridisation solution (0.5 M Na_2HPO_4, pH 7.2, 7% SDS) at 65 °C. Filters were washed first in solution 1 (40 mM Na_2HPO_4, pH 7.2, 5% SDS) and then solution 2 (40 mM Na_2HPO_4, pH 7.2, 1% SDS). Hybridisation was quantified using a phosphorimager (Bio-Rad). Southern blots were performed using the same protocol except with a 200-bp proximal promoter region (-162 to $+39$) as probe.

Sequence analysis. Human and murine sequence comparisons (dot-plot analysis) were performed using Gene Jockey II Sequence Processor (Biosoft). Putative transcription-factor-binding sites were identified using TFSearch.

Results

Isolation of the human *MCL-1* gene. A 6.5-kb *Eco*RI fragment, isolated from a human chromosome 1 library, as described above, was analysed by restriction mapping and subsequently sequenced (fig. 1A). This fragment encompassed the entire coding region of the *MCL-1* gene plus approximately 3.9 kb of 5′-flanking sequence (fig. 1B). The transcriptional start site was identified by primer extension analysis as an A, 80 residues upstream of the ATG translation initiation codon (fig. 1C). Comparison of the 5′-flanking region of this human gene with the murine counterpart [13] showed surprisingly little similarity (fig. 1D). Analysis of the 5′-flanking regions for potential transcription-factor-binding sites revealed consensus sequences as follows (relative to the transcription start site; equivalent murine sequences and positions shown in parentheses): STATx, $-92/-83$ ($-94/-85$); SRE, $-96/-86$ (no equivalent); Ets, $-118/-108$, $-222/-212$ ($-92/-82$, $-153/-143$); Sp1, $-167/-157$ ($-111/-102$); CRE-BP, $-252/-244$ ($-76/-67$); NF-κB, $-218/-208$ ($-117/-108$). Thus, although there is little overall sequence similarity in the human and murine promoters, some transcription-factor-binding sequences are common to both. We generated a mutant in which residue -218 was changed from G to A and residue -212 was changed from C to T. These mutations decreased the probability of an NF-κB site from 85% to 65% and yet this mutant showed no decreased reporter gene activity (data not shown). This raises doubt as to the presence of a genuine NF-κB site at residues $-218/-208$ in the human *MCL-1* gene.

Intron/exon structure of the gene. The human *MCL-1* gene contains two introns (fig. 2A). Exon 1 (768 bp) encodes 229 amino acids and the Arg:Arg motifs and PEST sequences that target the protein for proteolysis (fig. 2A). Also present in this exon is the BH3 domain. Exon 2 (248 bp) encodes 83 amino acids and the BH1 domain, while exon 3 encodes the carboxy-terminal 38 amino acids that contain the putative membrane-docking domain. The BH2 domain spans exons 2 and 3. There is a high level of similarity in the coding regions of both the human and murine *MCL-1* genes at the nucleotide level (fig. 2B), after comparison of the exon sequences of the human gene and the cDNA of the published mouse gene [13]. The 3′-untranslated region of the human and murine genes shows some similarity (fig. 2C).

Promoter analysis and reporter gene assays. The 5′-flanking region of the *MCL-1* gene was isolated and this and a series of constructs (generated by restriction enzyme digestion) were cloned in front of the luciferase reporter (fig. 3A). Constructs were transfected into U-937 cells (by electroporation) and cells were incubated for 22 h in the absence (control) or presence of PMA (10 ng/ml) or GM-CSF (100 U/ml). Luciferase reporter gene activity was then tested. Endogenous *MCL-1* promoter activity was detected for all reporter constructs (fig. 3B, C), with even the smallest construct containing only 142 bp of 5′-flanking sequence displaying promoter activity. The reporter activity of all constructs was increased by the addition of PMA (fig. 3B). Maximum

A

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ACTTCTCACTTCCGCTTCCTTCCAGTAAGGAGTCGGGGTCTTCCCCAGTTTTCTCAGCCAGGCGGCGGCGGCGACTGGCAATGTTTGGCCTCAAAAGAAA   100
                                                                                  M  F  G  L  K  R  N
CGCGGTAATCGGACTCAACCTCTACTGTGGGGGGGCCGGCTTGGGGGCCGGCAGCGGCGGCGCCACCCGCCCGGGAGGGCGACTTTTGGCTACGGAGAAG   200
  A  V  I  G  L  N  L  Y  C  G  G  A  G  L  G  A  G  S  G  G  A  T  R  P  G  G  R  L  L  A  T  E  K
GAGGCCTCGGCCCGGCGAGAGATAGGGGGAGGGGAGGCCGGCGCGGTGATTGGCGGAAGCGCCGGCGCAAGCCCCCCGTCCACCCTCACGCCAGACTCCC   300
 E  A  S  A  R  R  E  I  G  G  G  E  A  G  A  V  I  G  G  S  A  G  A  S  P  P  S  T  L  T  P  D  S
GGAGGGTCGCGCGGCCGCCGCCCATTGGCGCCGAGGTCCCCGACGTCACCGCGACCCCCGCGAGGCTGCTTTTCTTCGCGCCCACCCGCCGCGCGGCGCC   400
R  R  V  A  R  P  P  P  P  I  G  A  E  V  P  D  V  T  A  T  P  A  R  L  L  F  F  A  P  T  R  R  A  A  P
GCTTGAGGAGATGGAAGCCCCGGCCGCTGACGCCATCATGTCGCCCGAAGAGGAGCTGGACGGGTACGAGCCGGAGCCTCTCGGGAAGCGGCCGGCTGTC   500
  L  E  E  M  E  A  P  A  A  D  A  I  M  S  P  E  E  E  L  D  G  Y  E  P  E  P  L  G  K  R  P  A  V
CTGCCGCTGCTGGAGTTGGTCGGGGAATCTGGTAATAACACCAGTACGGACGGGTCACTACCCTCGACGCCGCCGCCAGCAGAGGAGGAGGAGGACGAGT   600
 L  P  L  L  E  L  V  G  E  S  G  N  N  T  S  T  D  G  S  L  P  S  T  P  P  P  A  E  E  E  E  D  E
TGTACCGGCAGTCGCTGGAGATTATCTCTCGGTACCTTCGGGAGCAGGCCACCGGCGCCAAGGACACAAAGCCAATGGGCAGGTCTGGGGCCACCAGCAG   700
L  Y  R  Q  S  L  E  I  I  S  R  Y  L  R  E  Q  A  T  G  A  K  D  T  K  P  M  G  R  S  G  A  T  S  R
GAAGGCGCTGGAGACCTTACGACGGGTTGGGGATGGCGTGCAGCGCAACCACGAGACGGCCTTCCAAGgtaaggggttcattaatcgccaaggcctcac   800
  K  A  L  E  T  L  R  R  V  G  D  G  V  Q  R  N  H  E  T  A  F  Q
tccctttttttccatctctccccggactcacccgccaagggtgggttggaaaccgaaacgagtcagtgttgaaacgtgtctcatcctattcctgaagccag   900

aatattctggccatgagtcattgtttccgcccatcttgattcttttggaaatggcagctcttgttcaaagaccggaaagggtgggatgtcaatttcaagt  1000

ggggtcaacctgagttctgtaaatcccagtagcgattttcccgccgcgggtgggcaggcgaatcttgcgccggtttagacaaaggaggccgtgaggacct  1100

gcatgcttttctttctcagGCATGCTTCGGAAACTGGACATCAAAAACGAAGACGATGTGAAATCGTTGTCTCGAGTGATGATCCATGTTTTCAGCGACG  1200
                    G  M  L  R  K  L  D  I  K  N  E  D  D  V  K  S  L  S  R  V  M  I  H  V  F  S  D
GCGTAACAAACTGGGGCAGGATTGTGACTCTCATTTCTTTTGGTGCCTTTGTGGCTAAACACTTGAAGACCATAAACCAAGAAAGCTGCATCGAACCATT  1300
G  V  T  N  W  G  R  I  V  T  L  I  S  F  G  A  F  V  A  K  H  L  K  T  I  N  Q  E  S  C  I  E  P  L
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  A  E  S  I  T  D  V  L  V  R  T  K  R  D  W  L  V  K  Q  R  G  W
agtggaagtagaatgaaggatttttttagagaggtggggatatctaaaggtttttatgacgcacggctgtttgcaggctctaactaaaggaccattgtt  1500

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aattcagttgaatactcttcagtggattcaaaccatgaaaaaataagtcaccaggggaggatagctgaaataattcctaaggcggtgcctgttttaatgg  1700

agaagatatggggtggagcctgcgttttaaacaaacccagatctgatgcaggatgtacttaactacgttgagaaaaactgatctgcgcaattgaggcgtt  1800

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gtttactattgctttaggggaaaccttagatattttaatttaccttctctctggatagtagtgttgttaagagagcagaaacccatacttgaaaatgtg  2100

cttttctttttgttttctagGATGGGTTTGTGGAGTTCTTCCATGTAGAGGACCTAGAAGGTGGCATCAGGAATGTGCTGCTGGCTTTTGCAGGTGTTG  2200
                      D  G  F  V  E  F  F  H  V  E  D  L  E  G  G  I  R  N  V  L  L  A  F  A  G  V
CTGGAGTAGGAGCTGGTTTGGCATATCTAATAAGATAGCCTTACTGTAAGTGCAATAGTTGACTTTTAACCAACCACCACCACCACAAAACCAGTTTAT  2300
A  G  V  G  A  G  L  A  Y  L  I  R  *
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GGGAAGAGTGCTCCCCATTGATTGAAGAGTCACTGTCTGAAAGAAGCAAAGTTCAGTTTCAGCAACAAACAAACTTTGTTTGGGAAGCTATGGAGGAGGA  2500

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GCCGAATT
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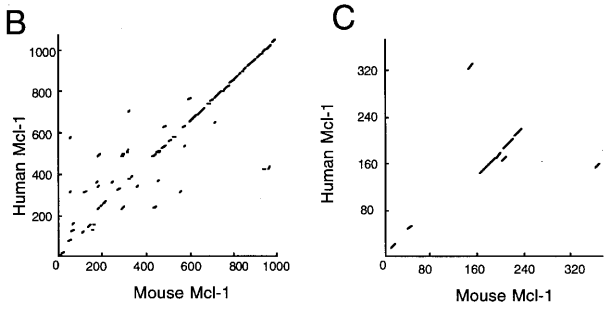

Figure 2. The human *MCL-1* coding regions showing intron:exon structure. (*A*) Nucleotide sequence of the human *MCL-1* gene, numbered from the transcription start site, with exon sequences shown in upper case and intron sequences shown in lower case. Amino acid residues comprising BH domains are boxed, with BH3 in exon 1, BH1 in exon 2 and BH2 spanning exons 2 and 3. The transmembrane (TM) domain is underlined. (*B*) Comparison of the coding regions of the human and mouse genes. (*C*) Comparison of the first 350 residues of the 3′-untranslated region.

activity was seen in a 294-bp fragment, but activity of this fragment was significantly decreased in a mutant that had a 47-bp deletion (between residues − 215 and − 168). GM-CSF could also induce a slight, but significant increase in *MCL-1* reporter gene activity (fig. 3C). In the 47-bp deletion mutant (− 215 to − 168), GM-

CSF-stimulated reporter gene activity was markedly decreased, indicating that this region may contain sequences regulating activated transcription via this cytokine. Reporter gene activity was actually decreased in the larger constructs (particularly construct 7). It is possible that transfection efficiency with this long plasmid was decreased, or the region −1659 to −3893 contains undefined regulatory sequences. PMA was shown to markedly increase *MCL-1* mRNA levels in U-937 cells after 3 h incubation, while GM-CSF stimulated a lower, but significant increase in mRNA levels (fig. 3D).

Discussion

There is much interest in understanding the molecular processes that control the expression of Mcl-1. While this protein can clearly play an important role in promoting cell survival, expression of Mcl-1 and Bcl-2 are

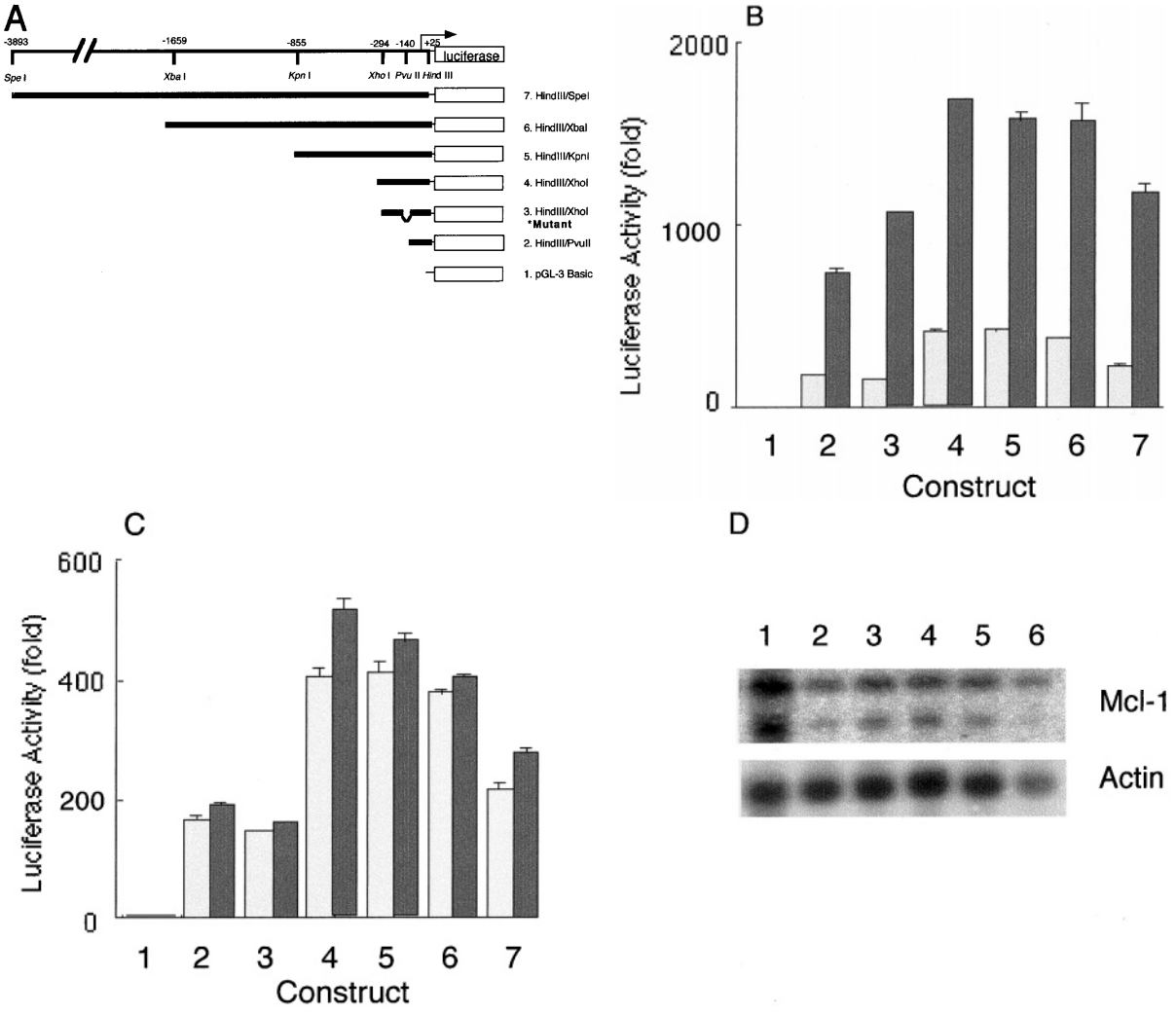

Figure 3. Reporter gene assays and Northern blots of *MCL-1* expression. (*A*) Schematic representation of the structure of six reporter constructs containing various parts of the *MCL-1* promoter. (*B*) Luciferase reporter gene activity after transfection of U-937 cells with these constructs, 24 h after stimulation with PMA (10 ng/ml): open bars show serum control, and closed bars show PMA-stimulated reporter activity. (*C*) Same as (*B*), except that the cells were stimulated for 24 h with 100 U/ml GM-CSF (closed bars) or serum control (open bars). (*D*) Northern blots of U-937 cells, with lanes loaded as follows: 1, cells treated for 3 h with 10 ng/ml PMA; 2, control, no additions; 3–6, 1, 3, 5 and 24 h after addition of 100 U/ml GM-CSF. Blots were probed with a cDNA representing residues 1236–2350 in the *MCL-1* transcript: after probing for *MCL-1* transcripts, filters were stripped and probed for actin mRNA.

apparently controlled by quite separate mechanisms. For example, cells like neutrophils do not express Bcl-2, but their survival appears to depend on expression of Mcl-1 [12]. Furthermore, there is often a reciprocal relationship between the expression of Mcl-1 and Bcl-2 in a variety of cells and tissues [8, 9, 17, 18]. Current ideas favour the proposal that Mcl-1 levels can be rapidly up-regulated by transcriptional activation and that this up-regulation of expression confers temporary resistance to apoptosis. Longer-term survival (e.g. of B cells) may then require subsequent activation of Bcl-2 expression [19–21]. There is also some indication that Bcl-2 and Mcl-1 may be located within distinct subcellular locations [11].

In this report, we showed that the major transcription start site is located 80 bp upstream of the translation initiation codon. This is some 10 residues further upstream than that reported previously [15], but in that report, no experimental data for this finding were shown. In the mouse gene, the major transcription start site is located 45 residues upstream of the initial ATG of the open reading frame [13]. In the mouse gene, the region between -197 and -69 contains cytokine (IL-3)-regulated elements, while in the human gene, a 162-bp 5′-flanking region was shown to possess PMA regulated elements [15]. This study showed that PMA-regulated elements are present within the first 142 residues upstream of the transcription start site, but greater unstimulated and stimulated promoter activity was seen in constructs containing 294 bp of the 5′-flanking region. The highest GM-CSF-regulated activity was also seen in this latter construct, but reporter activity in response to this cytokine was low compared to activity induced by PMA. Indeed, Northern analyses indicated that PMA treatment resulted in much higher levels of *MCL-1* mRNA compared with levels stimulated by GM-CSF. One of our reporter constructs contained a 47-bp deletion (fig. 1). Sequencing of this region revealed seven repeats of the motif GGCCCC, six of which are deleted in the mutant. Also absent in the mutant were two GCTCA repeats. These repeat elements were not present in the mouse promoter region [13]. In reporter gene constructs, deletion of this 47-bp region resulted in decreased activity in response to either serum (i.e. in cells incubated in medium containing bovine calf serum) or GM-CSF, with slightly decreased activity in response to PMA. No consensus TATA box was identified close to the transcriptional start site.

Sequencing of the coding regions of the gene and comparison with the published cDNA sequences (GenBank accession number: L08246) identified two introns and three exons. Introns possibly also exist in the 3′-untranslated region not present in our genomic clone. Exon 1 is the largest and contains the motifs that target the protein for its rapid proteolysis. Also present in this exon is the BH3 domain. BH1 was located in exon 2, while BH2 spans exons 2 and 3. The relatively short exon 3 is comprised mostly of the putative transmembrane-spanning domain.

As has been reported previously [14], the coding regions of the mouse and human *MCL-1* genes are remarkably similar ($>75\%$). What is somewhat surprising, therefore, is the relative lack of similarity in the 5′-flanking regions of the gene. Despite this apparent lack of similarity, both the mouse and human genes contain consensus motifs for certain transcription-factor-binding sites. Thus, despite the overall lack of sequence similarity, both the mouse and the human genes may be subject to regulation by common factors.

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