

# Myc–Max heterodimers activate a DEAD box gene and interact with multiple E box-related sites *in vivo*

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The c-Myc protein is involved in cell proliferation, differentiation and apoptosis through heterodimerization with Max to form a transcriptionally active sequence-specific DNA binding complex. By means of sequential immunoprecipitation of chromatin using anti-Max and anti-Myc antibodies, we have identified a Myc-regulated gene and genomic sites occupied by Myc–Max *in vivo*. Four of 27 sites recovered by this procedure corresponded to the highest affinity ‘canonical’ CACGTG sequence. However, the most common *in vivo* binding sites belonged to the group of ‘non-canonical’ E box-related binding sites previously identified by *in vitro* selection. Several of the genomic fragments isolated contained transcribed sequences, including one, MrDb, encoding an evolutionarily conserved RNA helicase of the DEAD box family. The corresponding mRNA was induced following activation of a Myc–estrogen receptor fusion protein (Myc–ER) in the presence of a protein synthesis inhibitor, consistent with this helicase gene being a direct target of Myc–Max. In addition, as for c-Myc, the expression of MrDb is induced upon proliferative stimulation of primary human fibroblasts as well as B cells and down-regulated during terminal differentiation of HL60 leukemia cells. Our results indicate that Myc–Max heterodimers interact *in vivo* with a specific set of E box-related DNA sequences and that Myc is likely to activate multiple target genes including a highly conserved DEAD box protein. Therefore, Myc may exert its effects on cell behavior through proteins that affect RNA structure and metabolism.

**Keywords:** chromatin immunoprecipitation/Myc-target genes/RNA helicase

## Introduction

The Myc oncogene encodes a basic helix–loop–helix leucine zipper (bHLH-LZ) transcription factor whose ability to bind to DNA with high affinity and to activate transcription is dependent upon dimerization with the bHLH-LZ protein Max (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991; Amati *et al.*, 1992; Kretzner *et al.*, 1992). The biological activities of Myc depend on

its ability to heterodimerize with Max, bind DNA and stimulate transcription (for review, see Amati and Land, 1994). Mutant Myc proteins deleted in the basic region, the LZ or the transactivation domain fail to transform or induce apoptosis (Stone *et al.*, 1987; Kato *et al.*, 1990; Evan *et al.*, 1992; Tikhonenko *et al.*, 1993). The requirement of Max for Myc function in transformation, transcription and apoptosis has been demonstrated by employing chimeric Myc and Max proteins that are unable to form heterodimers with their endogenous partners but bind efficiently to each other. The altered Myc proteins can homodimerize but are biologically inactive unless the appropriately altered Max is also present (Amati *et al.*, 1993a,b). Thus there is compelling evidence that Myc–Max complexes are crucial for Myc function.

The transcriptional activity of Myc–Max heterodimers is mediated by a high affinity recognition site, an E box element (CACGTG), as determined by sequential selection and amplification of binding sites (SAAB) (Blackwell *et al.*, 1990, 1993; Prendergast and Ziff, 1991). In addition, a set of variant E boxes containing fixed internal CG or TG dinucleotides has been found to bind Myc–Max heterodimers *in vitro* with lower apparent affinity than the CACGTG element (Blackwell *et al.*, 1993). These E box-related sequences, denoted as ‘non-canonical’ Myc–Max binding sites, include the following hexamers and one heptamer: CATGTG, CATGCG, CACGCG, CACGAG, CAACGTG.

In addition to possessing an activation domain, a region in the N-terminus of Myc, referred to as Myc box II (amino acids 122–140), has been linked to the ability of Myc to repress transcription of certain genes, a function that also appears to be necessary for cell transformation (Li *et al.*, 1994; Philipp *et al.*, 1994). This function may be independent of binding to Max and E boxes.

The realization that Myc–Max complexes function as transcription factors has prompted the search for ‘target’ genes directly regulated by the heterodimers. Identification of such genes is crucial for understanding the mechanism of Myc function. One strategy used a conditional Myc protein obtained by fusion of Myc to the ligand binding domain of the estrogen receptor (Myc–ER, Eilers *et al.*, 1989). In quiescent cells containing low levels of endogenous Myc, the Myc–ER protein can be activated by the addition of estrogens. This is sufficient to stimulate entry of the cells into the G<sub>1</sub>–S phase of the cell cycle. cDNA subtraction cloning applied in conjunction with the Myc–ER system resulted in identification of the first putative Myc target gene,  $\alpha$ -prothymosin (Eilers *et al.*, 1991). This gene has also been shown to possess a CACGTG site in its first intron which can mediate transcriptional activation by Myc, further supporting the notion that Myc directly regulates the rat  $\alpha$ -prothymosin gene (Gaubatz *et al.*, 1994).

Another putative Myc target, isolated using subtractive hybridization, is ECA39 which had been identified previously as a gene amplified in a teratocarcinoma cell line (Niwa *et al.*, 1990; Benvenisty *et al.*, 1992). Transcriptional induction of ECA39 by Myc is mediated by a CACGTG site located in the 5'-untranslated region of the gene. The function of  $\alpha$ -prothymosin and ECA39 proteins and their role in mediating Myc's effect on cell proliferation remain unknown. A major limitation of the cDNA subtraction approach is the fact that changes in gene expression consequent to Myc induction are often subtle and may not be detected. In addition, a large number of the genes identified by subtractive hybridization may not necessarily represent direct targets of Myc, but could be activated as a consequence of cell cycle entry.

Other Myc-Max target genes have been proposed based on their expression patterns and roles in cell proliferation. These include the ornithine decarboxylase gene (ODC) (Bello-Fernandez *et al.*, 1993). Recent experiments using the Myc-ER system have supported the hypothesis that the ODC gene is induced directly by Myc through two CACGTG sites present in the first intron of the mouse gene (Bello-Fernandez *et al.*, 1993; Wagner *et al.*, 1993; Tobias *et al.*, 1995). In addition, the ODC gene has been shown to induce transformation when overexpressed in cultured cells and to mediate Myc-induced apoptosis (Auvinen *et al.*, 1992; Packam and Cleveland, 1994).

Identification of Myc targets has occurred by searching for potential Myc-Max binding sites in gene regulatory regions. Putative Myc target genes identified in this manner include p53 (Reisman *et al.*, 1993) and the eukaryotic initiation factor 2 $\alpha$  (eIF-2 $\alpha$ ) (Rosenwald *et al.*, 1993b). However, the presence of a Myc-Max recognition element and its ability to mediate Myc transactivation when fused to a reporter gene is not sufficient to establish its identity as an *in vivo* Myc target gene. *In vivo* regulation of Myc target genes could be influenced by diverse factors such as chromatin structure and/or competition by more abundant transcription factors with similar DNA recognition specificity, such as USF, TFE3 and TFEB (Beckman *et al.*, 1990; Carr and Sharp, 1990; Gregor *et al.*, 1990). Most of the Myc-Max binding sites can also be recognized *in vitro* by USF and TFE3 and only a few, such as CATGCG and CAACGTG, appear to be recognized solely by Myc-Max (Blackwell *et al.*, 1993).

Here we describe the isolation of *in vivo* Myc-Max target genes by immunoprecipitation of chromatin with specific antibodies. Previous reports have adopted this approach to identify *Ultrabithorax* target genes in *Drosophila* and thyroid hormone-responsive genes in mammalian cells (Gould *et al.*, 1990; Bigler and Eisenman, 1994, 1995). Our results have led to the identification of a Myc-regulated DEAD box protein and revealed the sequence of several *in vivo* binding sites.

## Results

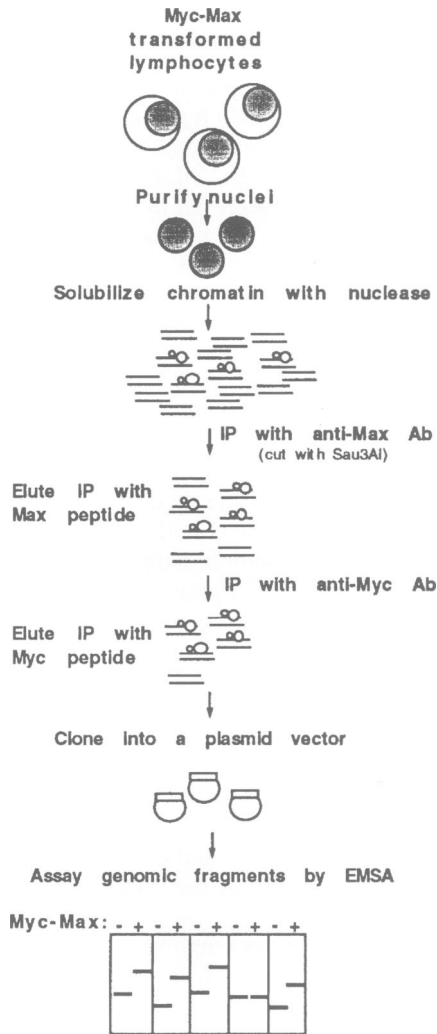
### Immunoprecipitation of Myc-Max chromatin complexes

The isolation of protein-DNA complexes from cells by means of antibodies directed against specific DNA binding proteins is dependent on the stability of the complexes under the conditions employed for extraction and immuno-

precipitation. For this study, an important consideration is whether the Myc-Max complexes could be solubilized from nuclei under conditions that maintained protein-DNA interaction. Only a small fraction of c-Myc protein is released from nuclei following hypotonic lysis. However, preliminary experiments indicated that quantitative release of Myc protein could be achieved by exposure of isolated intact nuclei to DNase I or micrococcal nuclease (see Materials and methods). If DNase digestion of nuclei at 4°C is allowed to proceed to complete solubilization of the nuclear chromatin, the majority of the Myc protein could then be released from nuclei under hypotonic conditions. An earlier study had also demonstrated the release of a fraction of v-Myc from nuclei following DNase treatment (Eisenman *et al.*, 1985). Since RNase digestion failed to render the majority of Myc protein soluble (Eisenman *et al.*, 1985; and data not shown), the DNase digestion results suggest that Myc and Max are bound to DNA or to structures stabilized by DNA under the cell lysis conditions employed. These observations suggested that Myc-Max heterocomplexes in association with DNA could be isolated from cells.

In order to enrich for Myc-Max-bound chromatin we employed two immunoprecipitation steps: first with anti-Max and then with anti-Myc antibodies. We had shown previously that both antibodies are highly specific (Hann *et al.*, 1988; Blackwood *et al.*, 1992). In addition, 'competitive' elution with the peptide immunogen was used to release Myc-Max complexes and their bound DNA from the antibodies. An outline of the procedure is shown in Figure 1.

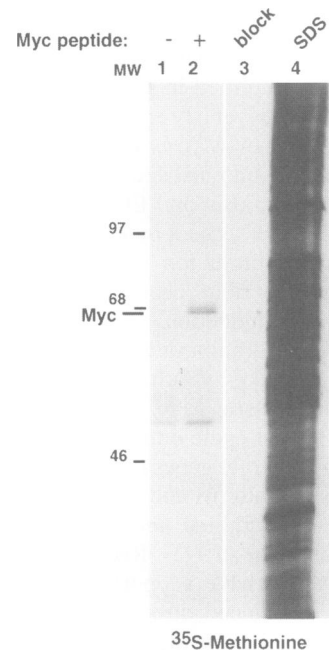
As a source of nuclei, we employed the cell line CB33/SVMyc-CMVMax, derived from Epstein-Barr virus-immortalized human cord blood lymphocytes, and transformed by introduction of Myc and Max genes (Gu *et al.*, 1993). These cells are tumorigenic and are thought to recapitulate the transformation process in Burkitt's lymphoma (Lombardi *et al.*, 1987). After hypotonic lysis of the cells, nuclei were isolated and exposed to DNase I (Experiment 1) or micrococcal nuclease (Experiment 2). The digestion conditions were established in order to achieve a chromatin fragmentation in the range of 0.5–3 kb. The soluble chromatin was released from nuclei by hypotonic lysis and immunoprecipitated with anti-peptide Max antiserum. After washing, the immunoprecipitate was subjected to restriction digestion with *Sau3A*I to facilitate the subsequent cloning step. Myc-Max complexes and their bound chromatin were eluted from the immune complex using the synthetic peptides initially used to prepare the antibodies. This minimized contamination by non-specifically bound DNA which would be released under more stringent elution conditions. The eluate was then immunoprecipitated with anti-Myc antibodies and Myc-Max chromatin complexes were eluted from the antibody using Myc-specific peptides. DNA was then extracted and cloned into a plasmid vector. Parallel analysis of the proteins labeled by [<sup>35</sup>S]methionine and immunoprecipitated with anti-Myc antibodies indicated that the peptide elution procedure efficiently released Myc from the immune complexes (Figure 2, lane 2), while the majority of background proteins remained on the protein A beads and were released with SDS (Figure 2, lane 3).



**Fig. 1.** Scheme of the chromatin immunoprecipitation procedure using anti-Max followed by anti-Myc antibodies. Details of the method are presented in Materials and methods.

Similar results were obtained with anti-Max immunoprecipitates from chromatin (data not shown).

The clones obtained either after a single cycle of immunoprecipitation with anti-Max antibodies, or after a second cycle with anti-Myc antibodies, were divided into pools of three or five clones and tested for the presence of Myc-Max binding sites in gel shift assays using bacterially expressed C92-Myc (Blackwood *et al.*, 1992) and baculovirus-produced Max (Ayer *et al.*, 1993). Among randomly chosen clones obtained after a single immunoprecipitation with anti-Max antibodies, only one out of 39 tested displayed binding to Myc-Max in electrophoretic mobility shift assays (EMSAs). This frequency was similar to the frequency of random DNA fragments obtained with control antiserum (0 out of 36; a fraction is shown in Figure 3A and B). However, a more substantial enrichment (13.3% in Experiment 1 and 13% in Experiment 2) of Myc-Max binding clones over the background (1.6% in Experiment 1 and 2.8% in Experiment 2) was achieved with a second cycle of immunoprecipitation using anti-Myc antibodies. Gel shift assays of a fraction of anti-Max- and anti-Myc-immunoprecipitated clones and the control immunoglobulin from Experiment 1 are shown in

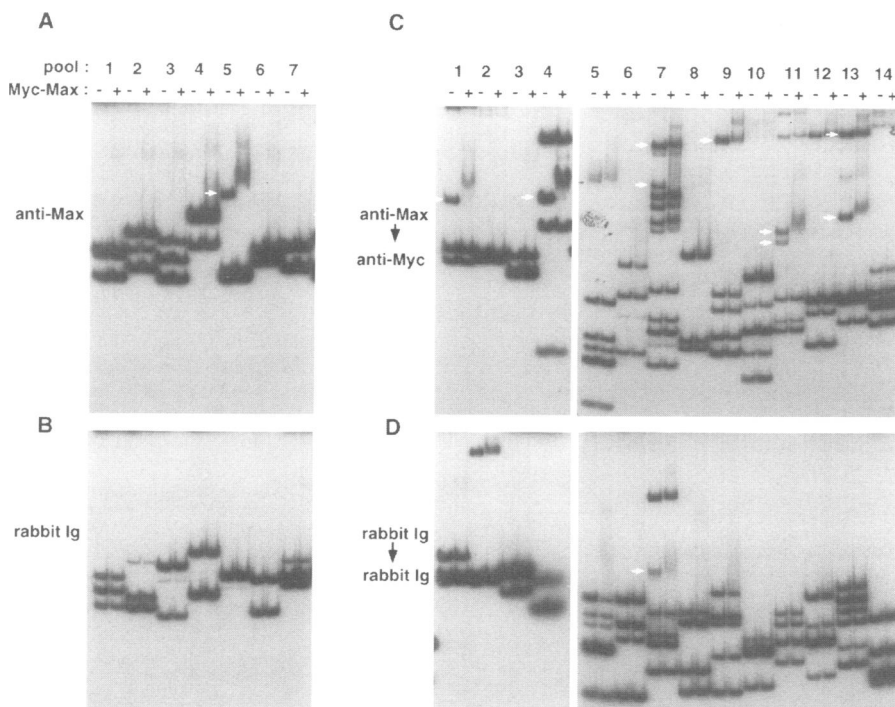


**Fig. 2.** Elution of Myc protein from chromatin immunoprecipitated with anti-Myc antibodies. Cells were metabolically labeled with [<sup>35</sup>S]methionine and processed as outlined in Figure 1 except that the soluble chromatin was subjected to a single cycle of immunoprecipitation with anti-Myc antibody (#1537). Myc was eluted with 2 µg of antibody-specific peptide (lane 2) or with SDS (lane 4). In lane 1, no peptide was added during the elution step and, in lane 3, the peptide was added to the antibody prior to the addition of the lysate.

Figure 3C and D. The results are summarized in Table I. The frequency of Myc-Max binding clones in the specifically immunoprecipitated DNA was calculated to be once per 2.2 kb (Experiment 1) and once every 1.1 kb (Experiment 2). This is in contrast with one binding clone every 12 kb in the non-specifically immunoprecipitated DNA (Table I). Therefore, we achieved an enrichment for Myc-Max binding clones ~5- to 10-fold over non-specifically immunoprecipitated DNA; this is a minimal estimate of the enrichment of specific Myc-Max binding hexamers since eight of the 20 clones sequenced contained more than one hexamer (see below).

***In vivo Myc-Max target sites***

Fourteen binding clones from Experiment 2 (referred to as MJ, ranging from ~150 to 850 bp) and nine from Experiment 1 (referred to as MM) were sequenced. MM11 (0.7 kb) and MM12 (0.4 kb) are subclones of original large clones (4.7 and 2.4 kb respectively) shown to possess Myc-Max binding sites by EMSA. Two clones from Experiment 2, MJ160 and MJ172, were found to be identical (only MJ172 is shown in Figure 4A). The sequences displayed in Figure 4A show that all but two contained one or more previously defined consensus Myc-Max binding sites (the complete sequences are available at EMBL, see accession numbers in Figure 4A legend). Two clones contained exclusively repetitive elements of the Alu family and probably represent non-specifically immunisolated DNA that contains binding sites (data not shown). Interestingly, the majority of sites corresponded to the 'non-canonical' Myc-Max binding sites, in contrast



**Fig. 3.** EMSA of labeled DNA inserts from a pool of clones obtained after the first cycle of immunoprecipitation with anti-Max antibody (A) or rabbit immunoglobulins (B) and after the second cycle with anti-Myc antiserum (C) or rabbit immunoglobulins (D). The white arrow indicates the fragments with altered mobility after addition of Myc and Max proteins (see Materials and methods). In this experiment, 5 ng of baculovirus Max and 15 ng of bacterial C92-Myc were added to the + lanes; an excess of BSA was present in both - and + lanes.

**Table I.** Enrichment of Myc-Max binding sites in the immunisolated DNA

	Total No. of clones	No. of clones tested	No. of binding clones	Frequency of Myc-Max sites	Average clone size	Average size of Myc-Max binding clones
Exp. 1						
Anti-Max	5760	248	33	1/2.2 kb	~0.3 kb	~3 kb
↓						
Anti-Myc						
Rabbit Ig	4640	242	4	1/12 kb	~0.2 kb	ND
↓						
Rabbit Ig						
Exp. 2						
Anti-Max	216 000	162	21	1/1.1 kb	~0.15 kb	~0.3 kb
↓						
Anti-Myc						
Rabbit Ig	20 800	138	4	1/12 kb	~0.35 kb	ND
↓						
Rabbit Ig						

Summary of the data obtained from recombinant clones derived by cloning one half of the immunoprecipitated DNA from Experiment 1 (Dnase I) and one quarter from Experiment 2 (micrococcal nuclease). The total number of clones refers to the number of recombinant clones as judged by interruption of the  $\beta$ -galactosidase gene that resulted in white bacterial colonies. The number of binding clones was determined by EMSA as described in Figure 3. The frequency of Myc-Max binding sites was calculated by dividing the number of binding clones by the added length of all the clones tested.

with the predominant *in vitro* selected canonical CACGTG sequence. About half of the clones contained sites, such as CATGCG and CAACGTG, that are not bound *in vitro* by USF and TFE3 (Blackwell *et al.*, 1993), indicating that some of these clones may contain highly specific Myc-Max recognition elements. Furthermore, a preference for flanking dinucleotides was observed (Figure 4B). Seventeen out of 27 sites had either a GC or CG on one side of the central hexamer and seven had a CT (Figure

4B). These preferences match the flanking dinucleotides observed in proposed Myc target genes (see Discussion). Several of these sequences, MJ19, MJ223, MJ143 and MJ255, when placed upstream of a minimal TK promoter, were able to mediate Myc transcriptional activation of a reporter CAT gene (Figure 5 and data not shown; Kretzner *et al.*, 1992). Depending on the ratio between expression vector and reporter, clone MJ19 activated 4- to 13-fold in the orientation that placed the CATGCG site closer to the



**Fig. 4.** (A) Partial DNA sequence of the Myc-Max binding clones obtained from experiment 1 (MM) and from experiment 2 (MJ) (see Table I) showing in bold the presumed core Myc-Max binding sites according to the described consensus (Blackwell *et al.*, 1993). Clones MJ19 and MJ195 contain two overlapping sites as indicated by the bracket extended to the flanking CG nucleotides. The size and EMBL accession number for each clone are as follows: MJ19, 127 bp, X99168; MM195, 162 bp, X99150; MJ223, 187 bp, X99151; MJ170, 136 bp, X99152; MM208, 148 bp, X99153; MJ66, 283 bp, X99154; MM109, 197 bp, X99155; MJ157, 542 bp, X99156; MJ172, 871 bp, X99157; MM174, 268 bp, X99158; MM11, 680 bp, X99159; MM12, 396 bp, X99160; MJ143, 219 bp, X99161; MJ159, 159 bp, X99162; MJ255, 377 bp, X99163; MJ238, 297 bp, X99164; MJ169, 158 bp, (pending); MM62, 366 bp, X99165; MJ155, 464 bp, X99166; MM 67, 168 bp, X99167. (B) Alignment of the binding sites with preferred flanking nucleotides shown in bold. The sequence from the rat  $\alpha$ -prothymosin first intron binding site (Gaubatz *et al.*, 1994), mouse ECA39 5'-non-coding region (Benvenisty *et al.*, 1993), mouse ODC site MB1 and MB2 in the first intron (Bello-Fernandez *et al.*, 1993) and human IF-2 $\alpha$  sites located upstream of the promoter (Humbelin *et al.*, 1989) are shown for comparison.

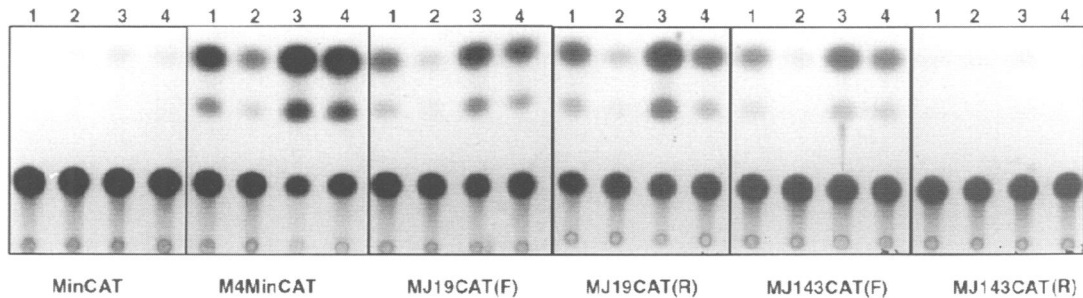
promoter (F) and only 3- to 6-fold in the opposite orientation (R) (Figure 5). Clone MJ143, containing a single CACGTG site, activated 3- to 16-fold when placed in the direction of transcription of its open reading frame (F), but failed to activate in the opposite orientation (R) (Figure 5). In the same assay, the positive control M4MinCAT reporter carrying a 4-fold repeat of the CACGTG site activated 4- to 9-fold (Figure 5). MJ172 did not activate transcription (possibly due to the large size of this clone, 871 bp, relative to the others, see legend of Figure 4A) and MJ169 and MJ162 were not tested (data not shown). These results indicate that several of the sequences isolated potentially mediate gene activation by Myc.

#### **An RNA helicase of the DEAD box family as a potential Myc target gene**

Thirty clones obtained from Experiment 1 that contained relatively large inserts (ranging from 2 to 12 kb) were tested as probes in Northern blots. Two short clones from Experiment 2, MJ143 (219 bp) and MJ255 (377 bp), were also used as probes because their DNA sequences revealed open reading frames with significant similarities to RNA- and DNA-dependent ATPases respectively (see below). However, only clones MM11 from Experiment 1 and

MJ143 from Experiment 2 hybridized to specific mRNAs from the original CB33/SVMyc-CMVMMax cells used in the immunoprecipitation experiments.

In order to characterize further clone MJ143, a full-length cDNA was obtained from a Burkitt's lymphoma library (referred to as MrDb for Myc-regulated DEAD box protein, see below). Sequencing of overlapping cDNAs hybridizing to the genomic clone MJ143 revealed that MrDb contained an open reading frame of ~1800 nucleotides encoding a protein with a maximal size of 615 amino acids (Figure 6, the full cDNA sequence including the 3'-untranslated region to be submitted to GenBank). The total length of overlapping cDNAs is 3130 bp, which corresponds to the size of the principal mRNA detected in human cells. Although the second and third methionines are in a good context for translation initiation (Kozak, 1986), there are no in-frame stop codons upstream of potential initiator methionines. It is possible, therefore, that the protein coding sequence is incomplete. The MrDb-encoded protein sequence contains motifs that are hallmarks of a family of RNA helicases possessing a DEAD box sequence (DEAD standing for Asp-Glu-Ala-Asp in the single letter amino acid code) (Figure 7, Linder *et al.*, 1989; Wassarman and Steitz, 1991; Schmid and Linder, 1992). This family of RNA helicases shares several



**Fig. 5.** Transcriptional responses in NIH3T3 cells of clones MJ19 and MJ143 placed upstream of the reporter construct MinCAT in the two possible orientations referred to as (F) and (R). The reporter construct is indicated under each panel; MinCAT and M4MinCAT were used as negative and positive controls respectively. Lanes 1 and 2 of all panels were transfected with 5 and 7.5  $\mu$ g of control empty expression vector pSP. Lanes 3 and 4 were transfected with 5 and 7.5  $\mu$ g of pSPMyc. The ratio of expression vector to reporter was 1:1 for lanes 1 and 3 and 3:1 for lanes 2 and 4.

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1  AATCAAACAAAAGCCCATGAATGTGGCTTATCAGAACTCAAATGGAGGCATGTCTCAA
   S K Q K P (M) N V G L S E T Q N G G (M) S Q
63  GAAGCAGTGGGAAATATAAAGTTACAAGTCTCCCCAGAAATCCACTGTATTACCAATGG
   E A V G N I K V T K S P Q K S T V L T N G
125  AGAAGCAGCAATGCAGTCTTCCAATTCAGAATCAAAAAGAAAAAGAAAAAGAGAAAAA
   E A A (M) Q S S N S E S K K K K K K K K
187  TGGTGAATGATGCTGAGCCTGATACGAAAAAGCAAAACTGAAAACAAGGAAATCTGAA
   (M) V N D A E P D T K K A K T E N K G K S E
249  GAAGAAAGTCCGAGACTACTAAAGAACAGAAAAATATGTGGAGAGCCAGATAATGATGA
   E E S A E T T K E T E N N V E K P D N D E
311  AGATGAGAGTGAAGTCCAGCTCCGCCCTGGGACTGACAGGAGCTTTGAGGATCATCTGT
   D E S E V P S L P L G L T G A F E D T S
373  TTGCTTCTCTATGTAATCTTGTCAATGAAACACTCTGAAGCAATAAAAAGAAATGGGTTT
   F A S L C N L V N E N T L K A I K E M G F
435  ACAAAACATGACTGAAATTCAGCATAAAAGTATCAGACCCTCTGGAAGGCCAGGGATCTCT
   T N M T E I Q H K S I R P L L E G R D L L
497  AGCAGCTGCAAAAACAGCAGTGGTAAACCCCTGGCTTTTCTCATCCCTGCACTGAACTCA
   A A A K T G S G K T L A F L I P A V E L
559  TTGTTAAGTTAAGGTTTCATGCCAGAAATGGAACAGAGCTTATTTCTCTACCTACTAGA
   I V K L R F M P R N G T G V L I L S P T R
621  GAAGTACCATGCAAACTTTGGTGTCTTAAAGAGCTGATGACTCACAGCTGATACCTA
   E L A M Q T F I G V L L K E L M T H H V H T Y
683  TGGCTTGATAATGGTGGCAGTAACAGATCTGCTGAAGCAGAACTTGGTAAATGGGATCA
   G L I M G G S N R S A E A Q K L G N G I
745  ACATCAITGGTGGCCACACAGCCGCTGTGCTGGACATATGCGAANTACCCAGAGTTATG
   N I M V A T P G R L D H M Q N T P G F M
807  TATAAAAACCTGCAGTGTCTGTTTATTGATGAAGCTGATGCTGATCTTGGATTTGGGTTTGA
   Y K N L Q C L V I D E A D R I L D V G F E
869  AGAGAAATTAAGCAAAATTTAAACTTTTGCCAACACGTAGACAGACTATGCTCTTTTCTG
   E L K Q I I K L L P F S
931  CCACCAAACCTGAAAAGTTGAAGACCTGGCAAGATTCTCTGAAAAGGAGCCATGTTAT
   A T Q T R K V E D L A R I S L K K E P L Y
993  GTTGGCGTTGATGATGATAAAGCAATGCAACAGCTGGATGGTCTTGAACAGGATATGTTGT
   V G V D D D K A N A T V D G L E Q G Y V V
1055  TTGTCCTCTGAAAAGAGATTCCTTCTGCTCTTTACATTCCTTAAGAAGAACCCGAAAGAGA
   C P S E K R F L L L P T F L K K N R K K
1117  AGCTTATGGTCTCTTTTTCATCTTGTATGCTGTGAAATACCACTATGAGTGTGCTGAAC
   K L M V F F S S C M S V K Y H Y E L L N Y
1179  ATTGATTTGCCCGCTTGGCCATTCATGAAAGCAAAAAGCAAAAATAGCGTACAACACATT
   I D L P V L A I H G K Q K Q N K R T T T F
1241  CTTCCAGTCTGCAATGCAGATTCGGGAACACTATTGTGTGTCGGATGTGCGCAGGAGAGAC
   F Q F C N A D S G T L L C T D V A A R G
1303  TAGACATTCCTGAAGTCCGACTGGATGTTTCAGTATGACCTCCGGATGACCTTAAGGAATAT
   L D I P E V D W I V Q Y D P D D P K E Y
1365  ATTCATCGTGTGGGTAGAAGCAGCCAGAGCCATAAATGGAGAGGGCATGCTTGTCTATTTT
   I H R V G R T A R G L N G R G H A L L I L
1427  GCGCCAGAAAGAAATGGGTTTCTCTGTTACTTAAACAATCCAAGTTCCATTAAAGTGAAT
   R P E E L G F L R Y L K Q S K V P L S E
1489  TTGACTTTTCTGGTCAAATTTCTGACATTCAGCTCAGCTTGAGAAATGATTGAAAG
   F D F S W S K I S D I Q S Q L E K L I E K
1551  AATTACTTTCTCATAAGTCAGCCAGGAAGCATATAAGTCATACATACGACCTATGATTC
   N Y F L H K S A Q E A Y K S Y I R A Y D S
1613  CCATCTCTGAAACAGATCTTAAATGTTAATACCTAAATTTGCTCAGGTTGCTCTGTCT
   H S L K Q I F N V N N L N L P Q V A L S
1675  TTGGTTCAAGGTGCCTCCCTTCGTTGATCTGACAGTCAACAGTAATGAAGGCAAGCAGAAA
   F G F K V P P F V D L N V N S N E G K Q K
1737  AAGCAGGAGGTTGGTGGTGGATTTGGCTACCAGAAAACCAAGAACTTGAGAAATCCAAAAT
   K R G G G G G F G Y Q I Q T K K K V E K I E K
1799  CTTTAAACACATTAGCAAGAAATCATCTGACAGCAGGAGTCTCTCACTGACACATGCTCT
   F K H I S K K S S D S R Q F S H
1861  TCCTTTCACTCTGAATAACTTTGCTCAAATAAATGAAATTTTTTCCCTTGTATTAACAGGAT
1923  TTTTGTAGACTTTTGAATTTGGACTTTACCTAACAAAGATATAAATGACTTGGGTGCAAGC

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**Fig. 6.** Partial DNA and derived protein sequence encoded by the MrDb cDNA (EMBL accession No. X98743). Potential initiator methionines are circled. The polylysine stretch is underlined with a broken line. The segment corresponding to the genomic clone initially isolated, MJ143, is underlined. The Myc-Max binding site is boxed.

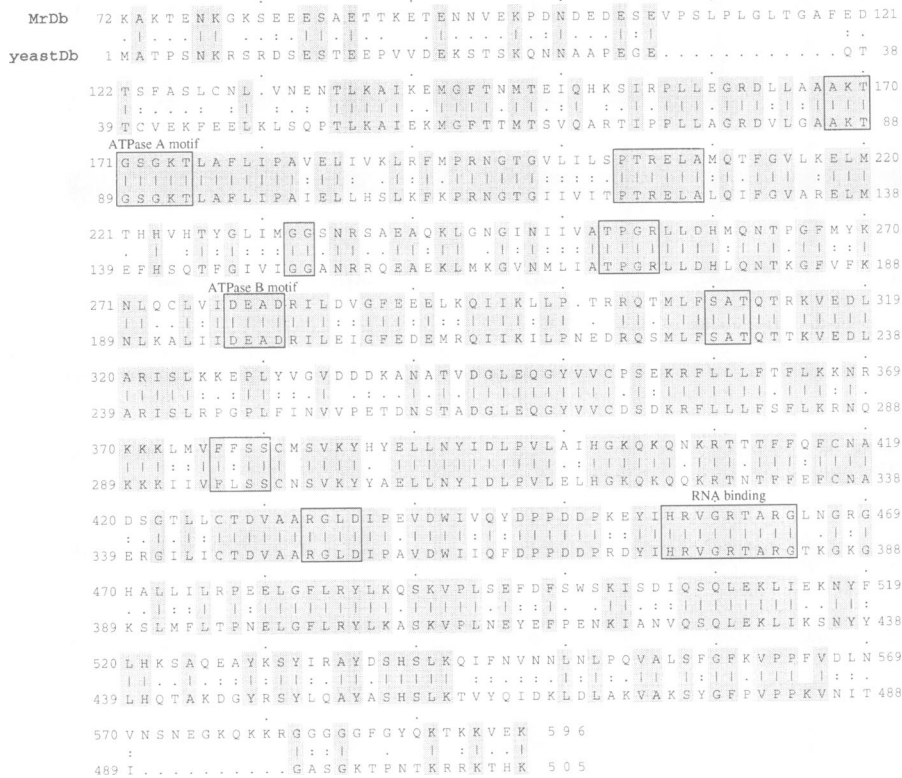
highly conserved domains which include a special version of the ATPase A and B motifs present in ATP binding proteins, the B motif corresponding to the DEAD box (see Figure 7). The conserved amino acids that characterize this class of proteins have been boxed according to Pause *et al.* (1993), including the RNA binding domain (Figure 7). The polylysine stretch at the N-terminus (underlined in Figure 6) may represent a nuclear localization signal (Dingwall and Laskey, 1991). MrDb was found to be most highly related (77% similarity) to a predicted yeast RNA helicase whose sequence was obtained from yeast chromosome 3 (GenBank accession No. S47451, communicated by R.Barrell; Figure 9).

The sequence corresponding to the genomic clone MJ143 is underlined on the cDNA sequence (Figure 7). Comparison of the genomic and cDNA sequences indicated multiple nucleotide differences, including two deletions of 3 and 2 bp respectively that shifted the open

reading frame corresponding to position +566 of MrDb and introduced a stop codon at +617. Sequencing of several cDNAs spanning this region revealed that they were all identical to MrDb. Only the MJ143 genomic sequence contained mutations, suggesting that the clone was probably derived from an MrDb pseudogene. Southern blot analysis with the MrDb probe also indicated the possible presence of two distinct genes (data not shown). Since both MJ143 and MrDb carry the Myc binding site, the probability of isolating either gene would be expected to be the same, assuming that Myc-Max occupies both sites (see Discussion). The potential regulation of MrDb mRNA by Myc was examined in a variety of systems as described below.

#### **MrDb mRNA is Myc regulated**

Rat-1 cells stably expressing a Myc-ER fusion protein have been shown to activate Myc functions in cell cycle



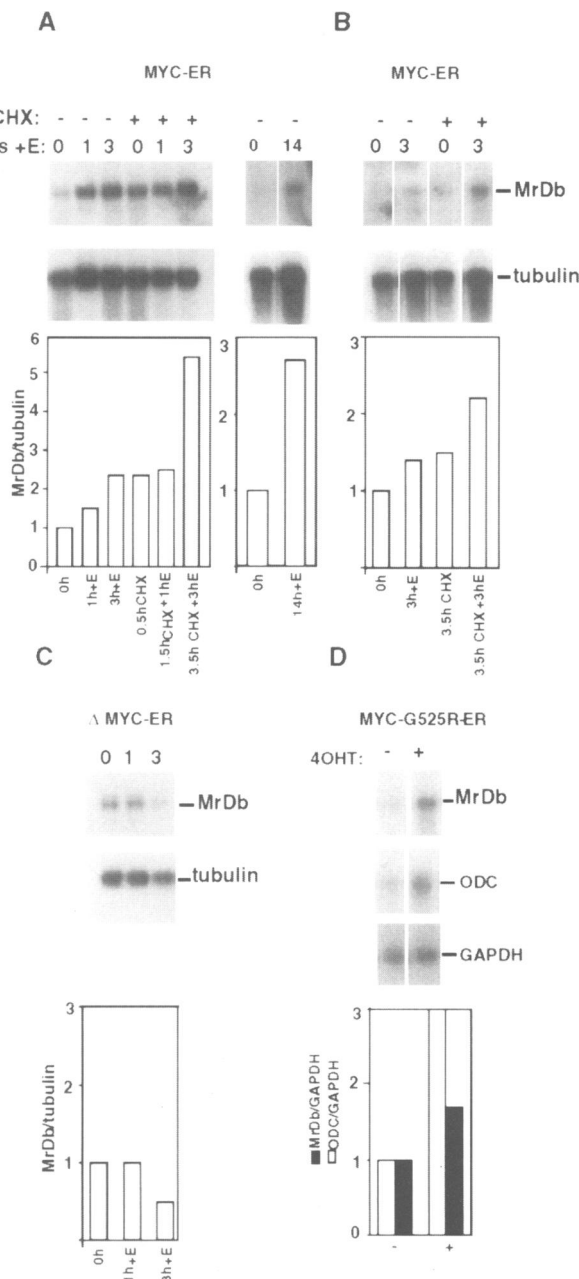
**Fig. 7.** Comparison of the human MrDb-encoded RNA helicase and its closest homolog, a yeast putative helicase (GenBank accession No. S47451, communicated by R.Barrell). The percent identity between these two proteins is 60% (highlighted in gray) and overall similarity is 77% as calculated by the Bestfit program (Genetics Computer Group, Madison, WI). The boxes pinpoint schematically the motifs conserved among all the members of the DEAD box proteins as previously reported by Pause *et al.* (1993).

entry and apoptosis upon addition of estrogen (Eilers *et al.*, 1989). We therefore probed Northern blots from Myc-ER cells at different times following estrogen exposure. The results are shown in Figure 8A. In serum-starved fibroblasts MrDb mRNA is induced 1 h after estrogen addition and increases ~2.5-fold after 3 h of induction. The levels remain high at 14 h after the addition of estrogen. A 2.5-fold increase is also detectable when cells are exposed to cycloheximide (CHX) prior to estrogen addition. Because CHX increased the basal levels of MrDb mRNA, we repeated the experiment by treating the cells for 3.5 h with either CHX alone or CHX plus estrogen (estrogen was added 30 min after CHX addition). The results shown in Figure 8B indicate that addition of estrogen increases MrDb levels above that induced by CHX alone. This suggests that Myc induction of MrDb is direct in that it does not require intermediate protein synthesis. As a control, we used a mutant Myc-ER chimera containing a deletion in the Myc transactivation domain (amino acids 106-143, ΔMyc-ER) (Penn *et al.*, 1990). Figure 8C shows that MrDb is not induced in the ΔMyc-ER cells after exposure to estrogen. In order to confirm that the increase of MrDb mRNA was due to Myc and not dependent upon the estrogen receptor transactivation domain, we performed Northern analysis on mRNA isolated from the cell line Myc-G525R-ER-Rat1 (Littlewood *et al.*, 1995; Solomon *et al.*, 1995). This cell line expresses a mutated Myc-ER protein that will respond to the estrogen analog 4-hydroxytamoxifen (4OHT) but is defective in the ER transactivation function. As shown in Figure 8D, MrDb mRNA and ODC are both induced

by 4OHT, confirming previous results for ODC (Wagner *et al.*, 1993), and indicating that their regulation is dependent on Myc and not on the ER portion of the chimeric protein.

The expression of MrDb mRNA was examined in other cell types where *c-myc* levels have been shown previously to be modulated during the transition from quiescence to proliferation. Serum stimulation of density-arrested WI38 primary human fibroblasts resulted in an ~3-fold increase in the expression of MrDb (Figure 9A). The increase was detectable at 3 h after serum addition and peaked at 5 h. In contrast, *c-myc* RNA levels peaked at 3 h. A few hours delay of MrDb mRNA relative to *c-myc* mRNA was a constant feature, consistent with its induction being a consequence of Myc expression. Interestingly, the expression pattern of the ODC gene is different. It increases rapidly after serum induction but peaks several hours later than MrDb and *myc* (Figure 9B, and Cosenza *et al.*, 1988). *max* was used as control for RNA loading variation since its levels have been shown to remain relatively constant during the cell cycle (Berberich *et al.*, 1992; Blackwood *et al.*, 1992).

We also measured MrDb RNA levels upon mitogenic stimulation of human primary B and T cells. Human B cells were isolated from human tonsils and stimulated with anti-μ antiserum as described (Clark *et al.*, 1989). Analysis of the total RNA indicated a 3.5-fold increase of MrDb RNA relative to *max* RNA within 3-5 h following stimulation (Figure 9C). Similarly, mitogenic stimulation of primary resting human T cells following PHA and



**Fig. 8.** Induction of MrDb mRNA in Rat 1 cells expressing the Myc-ER gene (A and B) or the Myc deletion mutant (amino acids 106–143) fused to the ER (C). Quiescent cells were treated with 17 $\beta$ -estradiol (250 nM) and poly(A)<sup>+</sup> RNA was extracted at the indicated time points. Cycloheximide was added when indicated at 10  $\mu$ g/ml 30 min before the addition of 17 $\beta$ -estradiol. In Rat-1 cells, the major mRNA detected with MrDb is 2.7 kb while it is 3 kb in human cells. The same blots were probed sequentially with MrDb and human tubulin. The plotted data were obtained after normalization to the tubulin signal. (D) Rat-1 cells expressing G525R mutant estrogen receptor-Myc fusion protein were treated with 250 nM hydroxytamoxifen for 7.5 h. The Northern blot of poly(A)<sup>+</sup> RNA was probed sequentially with rat MrDb, ODC and GAPDH. The plotted data were normalized for GAPDH.

PMA stimulation showed induction of MrDb within the first few hours (data not shown).

Myc mRNA and protein levels are known to be down-regulated during differentiation of numerous cell types, and in some cases the decrease in Myc has been shown to be required for differentiation (Meichle *et al.*, 1992).

We therefore analyzed the HL60 leukemia line during granulocyte differentiation following exposure to retinoic acid (Collins *et al.*, 1978; Westin *et al.*, 1982). Down-regulation of MrDb consistently followed the down-regulation of *c-myc* mRNA (Figure 10). Quantitation was relative to *max*, whose mRNA levels were shown previously to be constant upon retinoic acid treatment of HL60 cells (Luscher *et al.*, 1994). Differentiation of the HL60 cells was verified by probing the same blot with a cDNA encoding *CD18*, a gene up-regulated during retinoic acid differentiation (Figure 10, and Hickstein *et al.*, 1988).

These experiments demonstrate that the expression patterns of MrDb correlate with *c-myc* expression, while its induction in the Myc-ER system indicates that it is a direct *in vivo* target of Myc and Max.

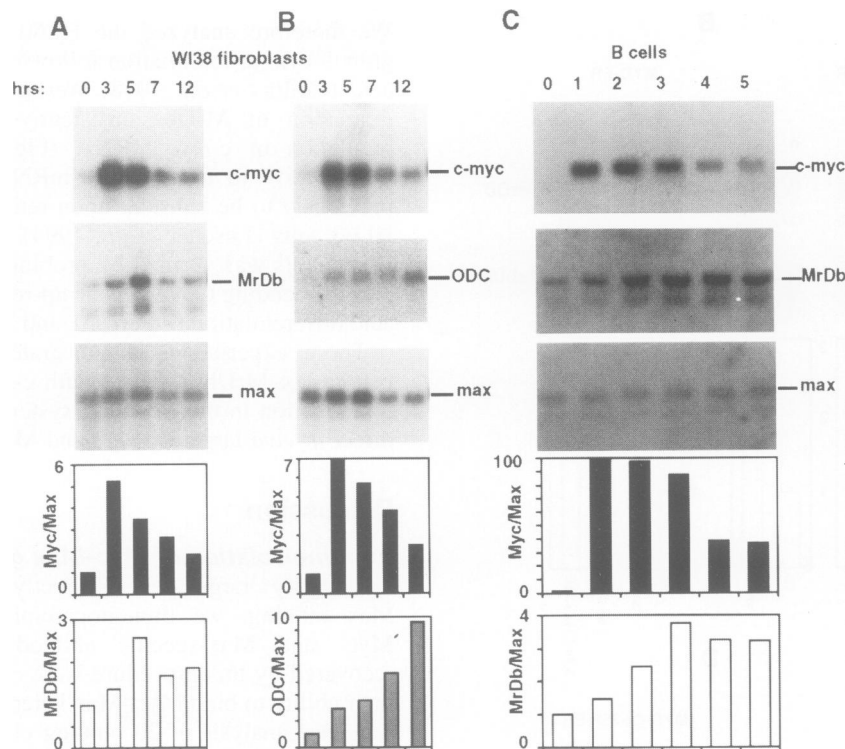
## Discussion

### Immunoisolation of Myc-Max genomic sites

To identify 'target genes' directly modulated by Myc-Max binding, we immunoprecipitated chromatin with Myc- and Max-specific antibodies. DNA fragments recovered by this procedure were cloned and assayed for their ability to bind Myc-Max heterodimers *in vitro*. DNA sequence analysis of 20 binding clones revealed that the majority contained specific variants of the CACGTG E box, previously defined as 'non-canonical' Myc-Max binding sites (Blackwell *et al.*, 1993). A number of the non-canonical sites had been shown to bind specifically Myc-Max and not USF or TFE3, two of the bHLH-ZIP proteins which recognize E box sequences (Blackwell *et al.*, 1993). These results suggest that the highest affinity *in vitro* binding site identified for Myc-Max heterodimers (CACGTG) does not represent the predominant *in vivo* binding site. Focusing on CACGTG as the primary Myc recognition element may therefore lead to the identification of genes which might well be targets of USF or the TFE family instead of Myc.

Interestingly, the binding sites identified revealed an apparent preference for specific flanking dinucleotides (see Figure 4B). Preference for specific flanking nucleotides had been reported in *in vitro* binding assays (Halazonetis and Kandil, 1991; Papoulas *et al.*, 1992; Blackwell *et al.*, 1993; Solomon *et al.*, 1993). However, these preferences were observed in the context of CACGTG as a core binding site while the preferences shown in Figure 4B may be related to the fact that they flank the 'non-canonical' sites present in most of the *in vivo* recovered fragments. Nonetheless, in agreement with Blackwell *et al.* (1993), we have never isolated the palindromic sequence GACCACGTGGTC, previously reported to be an optimal Myc-Max binding site (Halazonetis and Kandil, 1991). We observed an asymmetry in the conservation of flanking dinucleotides which closely matches binding sites found in the proposed Myc target genes  $\alpha$ -prothymosin, ECA 39, ODC and eIF 2 $\alpha$  (Figure 4B) (Humbelin *et al.*, 1989; Eilers *et al.*, 1991; Bello-Fernandez *et al.*, 1993). The crystal structure of Max homodimers bound to CACGTG did not demonstrate contacts with bases outside the hexamer (Ferré-D'Amare *et al.*, 1993). It is possible, therefore, that the asymmetrical conservation of flanking nucleotides reflects the ability of the Myc subunit to contact bases adjacent to the core hexamer. Indeed, different



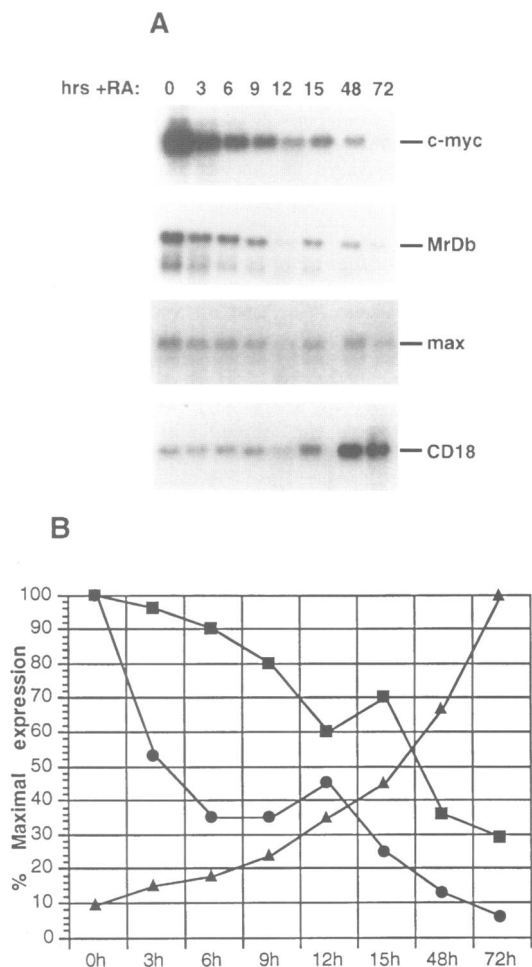


**Fig. 9.** MrDb mRNA levels correlate with Myc expression pattern. (A and B) Northern blot analysis of total RNA extracted from density-arrested WI38 primary human fibroblasts stimulated by the addition of fresh medium with 10% FCS. Blots were probed sequentially with human *c-myc*, MrDb, mouse ODC and human *max*. (C) Northern analysis of total RNA extracted from primary B cells isolated from human tonsils and stimulated with anti- $\mu$  antiserum. All the data plotted were obtained after normalization for *max* RNA.

preferences for flanking nucleotides was shown *in vitro* for Myc–Max versus Max–Max dimers (Solomon *et al.*, 1993). A requirement for specific flanking nucleotides by Myc–Max heterodimers might, in part, explain the discrepancy between our experimental finding of one binding site every 12 kb on the non-specific immunoprecipitated DNA in contrast with the predicted frequency of any of the five possible hexamers and one heptamer, that is  $5 \times (1/4^6 \text{ bp}) + 1 \times (1/4^7 \text{ bp}) = 1/780 \text{ bp}$ . This theoretical prediction has been confirmed by a computer search carried out on part of the available human genomic sequence covering  $8 \times 10^6 \text{ bp}$ . Therefore, Myc and Max do not bind to every single hexamer present in the genome. In addition to flanking nucleotides, Myc–Max may require close proximity of two hexamers, as detected in many of the clones sequenced (Figure 4A), or other as yet unknown sequences. It is likely that *in vivo* the Myc–Max available sites will be even more restricted due to DNA methylation, chromatin structure or other proteins bound to the complex that could determine specific flanking sequences. All these mechanisms may contribute to the ability of Myc–Max to regulate only a specific set of genes. Recently, the presence of a binding site for the transcription factor AP2 has been observed to overlap with the CACGTG hexamer in the  $\alpha$ -prothymosin and the ODC genes (Gaubatz *et al.*, 1995). The preferred GC dinucleotides that we found are part of the AP2 site present in the  $\alpha$ -prothymosin and ODC genes but they are not followed by a complete AP2 site in any of the clones sequenced. However, the majority of the hexamers are embedded within GC-rich sequences which could contain, at variable distances from the hexamers, potential binding sites for other transcription factors.

Recently, a functional role for flanking dinucleotides was demonstrated for another bHLH protein, MyoD (Weintraub *et al.*, 1994). It appears that the 2 bp flanking the  $\mu$ E5 E box present in the Ig enhancer prevent activation mediated by MyoD but not E12, thereby providing a rationale for the specificity of MyoD in the activation of only muscle-specific genes. In addition, specificity in the activation of different target genes by the yeast bHLH proteins PHO4 versus CPF-1 lies in the nucleotides flanking the shared core element (Fisher and Goding, 1992). Further experiments will be required to clarify the importance of the flanking dinucleotides for the ability of Myc–Max to specifically bind and/or mediate transactivation.

Our experiments indicate that the original genomic fragments corresponding to MrDb (MJ143) and the majority of the other binding sites tested can confer Myc-dependent transactivation when cloned upstream of a heterologous reporter gene (Figure 5, and data not shown). The CACGTG binding site for MrDb is located downstream from the promoter, within the coding region. Similarly, other Myc-responsive genes such as ODC, ECA39 and  $\alpha$ -prothymosin harbor binding sites either in 5'-untranslated regions or intron sequences (Eilers *et al.*, 1991; Benvenisty *et al.*, 1992; Bello-Fernandez *et al.*, 1993). It is interesting to note that *in vivo* cross-linking of homeodomain proteins to their target genes revealed binding not only at the promoter but also throughout the transcribed sequence, possibly due to the presence of low affinity sites throughout the genes (Walter *et al.*, 1994). It will be interesting to determine whether additional Myc binding sites are located in the promoter or intron sequences of the MrDb gene.



**Fig. 10.** The down-regulation of MrDb mRNA parallels *myc* RNA disappearance in the leukemia line HL60. (A) Total RNA was extracted at the indicated time points after exposure to 3  $\mu$ M retinoic acid. The same blot was hybridized sequentially to human *c-myc*, MrDb, human *max* and human CD18. (B) The data in the graph were normalized for *max* RNA. Squares correspond to MrDb/Max; circles to Myc/Max and triangles to CD18/Max.

#### Identification of a Myc-regulated cDNA

Screening of Northern blots revealed that only a small fraction of our immunisolated clones hybridized to mRNAs. This is to be expected in view of the fact that regulatory elements are usually located in non-transcribed sequences. Moreover, the presence of repetitive elements within the large genomic clones used to screen Northern blots could have obscured detection of low abundance mRNAs. Two clones hybridized to mRNAs: MJ143, whose binding site was within the protein coding sequence that we named MrDb, and clone MM11, which harbored a binding site ~300 bp downstream from a 1 kb stretch of transcribed sequence (data not shown). Sequence comparison of our isolated genomic DNAs with corresponding cDNAs indicated that both genomic clones MJ143 and MM11 were derived from pseudogenes. A potential explanation for this is the increased susceptibility of transcriptionally active chromatin to digestion by nucleases. Since pseudogenes are often silent, larger fragments would be generated from the pseudogenes relative to the transcribed gene. Due to the restriction digestion prior to cloning (see Figure 1), it is possible that fragments

<250 bp would not be cut twice and therefore would not be cloned. This could account for our biased isolation of the non-transcribed genes in cases where the binding site is within or near the transcribed sequence.

We focused on MrDb and examined its expression pattern because the Myc-Max binding site, shown to mediate transcriptional activation by Myc (Figure 5), was conserved in the transcribed gene. Our results show that MrDb is inducible in the Myc-ER system (Eilers *et al.*, 1989). Inducibility is independent of protein synthesis and thus consistent with the possibility that transcriptional activation is mediated by the pre-existing Myc fusion protein (Figure 8). Moreover, MrDb mRNA is induced within 3–5 h following mitogenic stimulation of primary human fibroblasts and B cells (Figure 9). The timing of MrDb induction parallels the increase in *c-myc* RNA levels. However, we do detect low levels of MrDb mRNA in quiescent cells. It is possible that constitutive transcription factors maintain MrDb basal expression while the function of Myc-Max would be to boost its expression upon cell cycle entry.

MrDb mRNA decreases during differentiation of HL60 (Figure 10) and primary keratinocytes (Hurlin *et al.*, 1994), consistent with the disappearance of *c-myc* RNA. The down-regulation of MrDb during differentiation suggests that MrDb could be a target for repression by Mad proteins, a family of bHLH-LZ proteins that associate with Max, are induced upon differentiation in various cell types and act as transcriptional repressors (Ayer and Eisenman, 1993; Ayer *et al.*, 1993; Hurlin *et al.*, 1994, 1995; Larsson *et al.*, 1994)

#### A DEAD box protein as a Myc target

The protein encoded by MrDb shows a striking similarity to a yeast RNA helicase of the DEAD box family. This class of highly conserved proteins has the ability to bind and unwind single-stranded RNA in an ATP-dependent fashion (for reviews, see Linder *et al.*, 1989; Wassarman and Steitz, 1991; Schmid and Linder, 1992). Members of the DEAD box family, such as the eukaryotic initiation factors 4AI and II, are involved in modulating translation efficiency of specific mRNAs (Nielsen and Trachsel, 1988) and RNA stability in bacteria (Iost and Dreyfus, 1994). Others are involved in RNA splicing (Wassarman and Steitz, 1991). In humans, a few RNA helicases of the DEAD box family have been cloned. One is p68, a nuclear helicase expressed exclusively in proliferating cells that shares an epitope with the SV40 T antigen (Ford *et al.*, 1988). It is possible that MrDb encodes a nuclear RNA helicase since there is a potential nuclear localization signal (KKKKKKKRK) in the N-terminus of the protein. Therefore, MrDb protein could play a role in the stability or transport of specific RNAs involved in cell growth. Post-transcriptional regulation of serum-induced genes by overexpression of Myc has indeed been described previously (Prendergast and Cole, 1989). Also down-regulation of the integrin LFA-1 by Myc seems to involve both a transcriptional as well as a post-transcriptional mechanism (Inghirami *et al.*, 1990). Interestingly, overexpression of eIF-4E, a subunit of the mRNA cap binding complex that has an RNA helicase-associated function, results in cell transformation and is accompanied by increased translation efficiency of RNAs such as cyclin

D1 (Lazaris-Karatzas *et al.*, 1990; Rosenwald *et al.*, 1993a). A role for RNA helicases in tumorigenesis has been suggested by the finding of a translocation in a B cell lymphoma involving the RCK1 gene encoding a DEAD box protein (Akao *et al.*, 1992; Lu and Yunis, 1992). Also another family member, DDX1, is co-amplified and overexpressed with the *N-myc* gene in a subset of retinoblastoma and neuroblastoma lines as well as in primary neuroblastomas (Godbout and Squire, 1993; Manohar *et al.*, 1995; Squire *et al.*, 1995).

The correlation of MrDb gene expression levels with *c-myc* mRNA together with the presence of a Myc–Max binding site capable of mediating Myc transcriptional activation are consistent with MrDb being an *in vivo* Myc target gene. The role of MrDb as a mediator of Myc biological functions remains to be established and we are currently pursuing such experiments. It is likely that the ability of Myc to trigger cell proliferation will be mediated by multiple genes, and the genomic sequences we have isolated may provide a means of identifying a complete set of Myc–Max target genes.

## Materials and methods

### Preparation of chromatin and immunoprecipitations

For each immunoprecipitation experiment, 2 l of exponentially growing CB33/SVMyc-CMVMax cells (Lombardi *et al.*, 1987) ( $\sim 10^9$ ) were washed once in phosphate-buffered saline (PBS) supplemented with 0.5 g of MgCl<sub>2</sub> per 500 ml, swollen for 10 min in 10 ml of hypotonic buffer [10 mM Tris–HCl pH 7.4, 10 mM KCl, 1 mM dithiothreitol (DTT)] and lysed with 30 strokes of a Dounce homogenizer (A pestle). All manipulations, including lysis, antibody incubations and enzyme digestions were performed at 4°C and in the presence of protease inhibitors. Nuclei were recovered by centrifugation at 2000 g for 10 min and washed once by resuspending in nuclear buffer A (15 mM Tris pH 7.4, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 0.3 M sucrose, 1 mM DTT). Nuclei in 10 ml of buffer A were then exposed either to 20 U/ml of DNase I (Worthington Biochem.) in the presence of 1 mM CaCl<sub>2</sub> for 15 min at 4°C (Experiment 1) or to 400 U/ml of micrococcal nuclease (Worthington Biochem.) in buffer A with 10% glycerol (Experiment 2). Buffers and digestion conditions were adapted from previously published procedures aimed at preserving chromatin structure (Hewish and Burgoyne, 1973; Bellard *et al.*, 1989; Wu, 1989; Gould *et al.*, 1990). The digestions were stopped with 2.5 mM EGTA and 10 mM EDTA. The soluble chromatin fraction was obtained by lysis and homogenization of the nuclear pellet in 6 ml of hypotonic TEP buffer (12 mM Tris-base neutralized with 3 mM EDTA-free acid), and by recovering the supernatant after spinning at 2000 g for 5 min. This fraction was immediately adjusted to 100 mM KCl, 10 mM Tris–HCl pH 7.4, and bovin serum albumin (BSA, molecular biology grade, Sigma) to 0.5 mg/ml. The soluble chromatin was pre-cleared with 0.2 ml of protein A–Sepharose beads (blocked with BSA) for 20 min followed by clarification at 16 000 g for 10 min in a Sorvall swinging bucket rotor. The supernatant was either incubated with protein A–Sepharose containing 20–40 µg of affinity-purified rabbit anti-Max antiserum (#7902, Blackwood *et al.*, 1992) or with rabbit immunoglobulins as control. After 1 h incubation, the immunoprecipitates were washed on a column with 5 ml of buffer A supplemented with 2.5 mM EGTA and 5 mM EDTA. The column was then equilibrated with *Sau3AI* digestion buffer and bound DNA was digested with 160 U of enzyme (New England Biolabs) for 1 h at 4°C. The column was washed with 5 ml of buffer A supplemented with 150 mM KCl followed by 5 ml of buffer A. Myc–Max–DNA complexes were eluted from the antibody column by exposure for 15 min to 1 ml of buffer A containing 100 µg of antibody-specific Max peptide. A fraction of the eluate was saved for analysis, the rest was adjusted to 0.5 mg/ml BSA and further immunoprecipitated with anti-Myc antiserum (#1537, Blackwood *et al.*, 1992) as described above. The second eluate obtained with an antibody-specific Myc peptide was adjusted to 0.5% SDS, 100 µg of proteinase K and digested at 37°C overnight. After two phenol–chloroform extractions, the DNA was precipitated and ligated into the plasmid vector,

PVZ1 (Henikoff and Eghtedarzadeh, 1987) at the *Bam*HI site. A fraction of the ligation was transformed into highly competent *Escherichia coli* (Epicurian E.Coli XL-1 Blue, Stratagene) or DH5 $\alpha$  MCR (Gibco, BRL). For protein analysis,  $\sim 5 \times 10^8$  CB33 cells/SVMyc-CMVMax were labeled with [<sup>35</sup>S]methionine as described (Blackwood *et al.*, 1992). Cell lysis and immunoprecipitations were as described for the recovery of DNA except that, after a first cycle of immunoprecipitation with anti-Myc, the eluate was concentrated (Wessel and Flugge, 1984) and analyzed by SDS–PAGE.

### Screening of recombinant clones by electrophoretic mobility shift assay

Pools of three to five colonies were used to prepare miniprep DNA. One quarter was digested with *Eco*RI and *Hind*III and labeled with Klenow. An aliquot was used for each gel shift reaction in 10 µl of HMO.1 buffer containing 150 mM KCl (25 mM HEPES pH 7.5, 150 mM KCl, 10 mM MgCl<sub>2</sub>, 0.1% NP-40, 0.5 mg/ml BSA, 10% glycerol, 1 mM DTT) and incubated at room temperature for 30 min in the presence or absence of 5–10 ng of baculovirus Max (Ayer *et al.*, 1993) and 25 ng of GST–C92Myc (Blackwood and Eisenman, 1991). Heterodimers between Myc and Max were formed by incubating the undiluted proteins at room temperature for 10 min. Myc–Max–DNA complexes were resolved by 4% acrylamide gels run with 25 mM HEPES pH 7.5 in the gel and in the running buffer. Electrophoresis was for 2.5–3 h at 150 V at 4°C. Shifts of fragments >700 bp were hard to distinguish from non-specific retardation and were disregarded.

### Sequencing and cDNA cloning

The DNA sequence of individual clones was obtained using an Applied Biosystems automated apparatus with the M13 forward and reverse primers.

cDNAs corresponding to the genomic clone MJ143, referred as MrDb, were obtained by screening a cDNA library from a Burkitt lymphoma cell line, Manca (gift from Lynn Graf, Fred Hutchinson Cancer Research Center, Seattle), prepared according to Nottenburg *et al.* (1990). The probe spanned the entire MJ143 clone and was labeled by PCR using internal primers. Hybridizations were carried out in 0.5 M sodium phosphate buffer pH 6.8, 1 mM EDTA, 7% SDS and 100 µg/ml denatured salmon sperm DNA at 65°C. Hybond N filters (Amersham) were washed according to the manufacturer, including the high stringency wash at 65°C in 0.1× SSPE. The majority of the open reading frame of MrDb cDNA was derived from two overlapping clones. The 5' end of the coding region was obtained by amplifying an aliquot of the phage stock with a nested primer and a primer in the phage vector. The 5' end-amplified DNA was cloned into the PCRII vector (Stratagene) and screened by hybridization. The clones containing the longest inserts were used to probe the cDNA library again. We obtained several different clones, and the largest clone extended the 5'-coding region of ~300 bp. The entire reconstructed cDNA is 3130 bp and matches the size of the major mRNA detected in Northern blot in human cells. The full-length cDNA was sequenced on both strands using progressive exonuclease III deletions as described (Henikoff, 1984).

A rat cDNA clone corresponding to MrDb was obtained by screening a GH4 cell cDNA library with the MrDb coding region; its identity was verified by partial sequence analysis (Bigler and Eisenman, 1995).

### CAT assays

The genomic clones MJ19, MJ143, MJ223, MJ170 and MJ172 were amplified by PCR using M13 forward and reverse primers present in the cloning vector PVZ1, cut with *Sau3AI* and subcloned at the *Bam*HI site of the CAT reporter plasmid MinCAT (Kretzner *et al.*, 1992). MJ255 was subcloned directly using *Bam*HI from PVZ1 into MinCAT. Myc expression was under the control of an SV40 promoter (pSP). NIH3T3 cells were transfected with a total of 10 µg of DNA as indicated in the figure legends. Cell extracts were normalized for transfection efficiency by the inclusion of a  $\beta$ -galactosidase expression plasmid (pCH11, Pharmacia) and subsequent  $\beta$ -galactosidase assays.

### Northern analysis and probes

Preparation of total RNA, poly(A)<sup>+</sup> RNA and Northern blot were performed by standard techniques (Ausubel, 1994). Hybridization conditions were as described for the library screening, with the exception of the Rat1–Myc–ER-derived RNA which was analyzed at lower stringency. Hybridizations were at 58°C and washes at 50°C.

Control probes for RNA loading variations were human  $\alpha$ -tubulin (Cowan *et al.*, 1983) and human GAPDH (Tokunaga *et al.*, 1987). The *c-myc* probe was a 0.8 kb *Clal*–*Eco*RI fragment from a human clone

(Hann *et al.*, 1988); *max* was 0.55 kb of human cDNA (Blackwood and Eisenman, 1991); ODC was 1.6 kb of a mouse cDNA corresponding to a *Sall*-*PvuII* fragment (Kahana and Nathans, 1984); CD18 was a 1.8 kb *EcoRI* fragment from a human cDNA containing exons 7–16 (Hickstein *et al.*, 1988)

### Cell culture

CB33 cells expressing exogenous Myc and Max from episomal vectors (SVMyc and CMVMax, Gu *et al.*, 1993) were maintained in Iscove's medium with 10% fetal calf serum (FCS, Gibco), 700 µg/ml of neomycin (Sigma) and 300 µg/ml of hygromycin (Calbiochem). Rat-1 cells expressing the Myc-ER chimera were cultured in phenol red-free Dulbecco's modified medium in the presence of 10% charcoal-treated FCS (Hyclone). For synchronization experiments, confluent cells were starved for 24 h in 0.1% serum. 17β-Estradiol (Sigma) was added to a final concentration of 250 nM. When indicated, CHX was added at 10 µg/ml. Rat-1 cells expressing the G525R mutant estrogen receptor fused to Myc (provided by M.Eilers) were cultured in regular Dulbecco's modified medium containing 10% FCS. Cells were synchronized by density arrest and serum starvation at 0.1% for 48 h. Stimulation was with 250 nM 4OHT (gift from M.Eilers). Primary B cells were obtained from human tonsils, and proliferative stimulation was carried out by addition of anti-µ antibody at 5 µg/ml (Clark *et al.*, 1989). W138 cells were purchased from the American Tissue Type Collection and cultured in Dulbecco's modified medium supplemented with 10% FCS. For induction of quiescence, cells were seeded at  $2 \times 10^4/\text{cm}^2$  and medium was not changed for 11 days as described (Cosenza *et al.*, 1988). Fresh medium was added, and cell cycle progression was monitored by propidium iodide staining of fixed cells. RNA was collected at the indicated time points. HL60 cells (kindly provided by S.Collins, Collins *et al.*, 1978) were cultured in RPMI medium in the presence of 5% FCS. For differentiation experiments, exponentially growing cells were exposed to 3 µM retinoic acid. Expression of the CD18 cell surface marker (Hickstein *et al.*, 1988) and down-regulation of Myc were used as differentiation parameters (Westin *et al.*, 1982).

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### References

Akao, Y., Seto, M., Yamamoto, K., Iida, S., Nakazawa, S., Inazawa, J., Abe, T., Takahashi, T. and Ueda, R. (1992) The RCK gene associated with t(11:14) translocation is distinct from the MLL/ALL-1 gene with t(4:11) and t(11:19) translocations. *Cancer Res.*, **52**, 6083–6087.

Amati, B. and Land, H. (1994) Myc-Max-Mad: a transcription factor network controlling cell cycle progression, differentiation and death. *Curr. Opin. Genet. Dev.*, **4**, 102–108.

Amati, B., Dalton, S., Brooks, M.W., Littlewood, T.D., Evan, G.I. and Land, H. (1992) Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. *Nature*, **359**, 423–426.

Amati, B., Brooks, M.W., Levy, N., Littlewood, T.D., Evan, G.I. and Land, H. (1993a) Oncogenic activity of the c-Myc protein requires dimerization with Max. *Cell*, **72**, 223–245.

Amati, B., Littlewood, T.D., Evan, G.I. and Land, H. (1993b) The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. *EMBO J.*, **12**, 5083–5087.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Scidman, J.G., Smith, J.A., Struhl, K. (eds), (1994) *Current Protocols in Molecular Biology*. John Wiley & Sons, NY, USA.

Auvinen, M., Paasinen, A., Andersson, L.C. and Hölttä, E. (1992) Ornithine decarboxylase activity is critical for cell transformation. *Nature*, **360**, 355–358.

Ayer, D. and Eisenman, R.N. (1993) A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. *Genes Dev.*, **7**, 2110–2119.

Ayer, D.E., Kretzner, L. and Eisenman, R.N. (1993) Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. *Cell*, **72**, 211–222.

Beckmann, H., Su, L.K. and Kadesch, T. (1990) TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer mE3 motif. *Genes Dev.*, **4**, 167–179.

Bellard, M., Dretzen, G., Giangrande, A. and Ramain, P. (1989) Nuclease digestion of transcriptionally active chromatin. *Methods Enzymol.*, **170**, 317–347.

Bello-Fernandez, C., Packam, G. and Cleveland, J.L. (1993) The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc. Natl. Acad. Sci. USA*, **90**, 7804–7808.

Benvenisty, N., Leder, A., Kuo, A. and Leder, P. (1992) An embryonically expressed gene is a target for c-Myc regulation via the c-Myc binding sequence. *Genes Dev.*, **6**, 2513–2524.

Berberich, S.J., Hyde-De-Ruyscher, N., Espenshade, P. and Cole, M.D. (1992) Max encodes a sequence-specific DNA-binding protein and is not regulated by serum growth factors. *Oncogene*, **7**, 775–779.

Bigler, J. and Eisenman, R.N. (1994) Isolation of a thyroid hormone-responsive gene by immunoprecipitation of thyroid hormone receptor-DNA complexes. *Mol. Cell. Biol.*, **14**, 7621–7632.

Bigler, J. and Eisenman, R.N. (1995) Novel location and function of a thyroid hormone responsive element. *EMBO J.*, **14**, 5710–5723.

Blackwell, T.K., Kretzner, L., Blackwood, E.M., Eisenman, R.N. and Weintraub, H. (1990) Sequence-specific DNA binding by the c-Myc protein. *Science*, **250**, 1149–1152.

Blackwell, T.K., Huang, J., Ma, A., Kretzner, L., Alt, F.W., Eisenman, R.E. and Weintraub, H. (1993) Binding of Myc proteins to canonical and noncanonical DNA sequences. *Mol. Cell. Biol.*, **13**, 5216–5224.

Blackwood, E.M. and Eisenman, R.N. (1991) Max: a helix-loop-helix leucine zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science*, **251**, 1211–1217.

Blackwood, E.M., Lusher, B. and Eisenman, R.N. (1992) Myc and Max associate *in vivo*. *Genes Dev.*, **6**, 71–80.

Carr, C.S. and Sharp, P.A. (1990) A helix-loop-helix protein related to the immunoglobulin E-box binding proteins. *Mol. Cell. Biol.*, **10**, 4384–4388.

Clark, E.A., Shu, L.G., Lüscher, B., Draves, K.E., Banchereau, J., Ledbetter, J.A. and Valentine, M.A. (1989) *J. Immunol.*, **143**, 3873–3880.

Collins, S.J., Ruscetti, F.W., Gallagher, R.E. and Gallo, R.C. (1978) Terminal differentiation of human promyelocytic leukemia cells induced with dimethyl sulphoxide and other polar compounds. *Proc. Natl. Acad. Sci. USA*, **75**, 2458–2462.

Cosenza, S.C., Owen, T.A., Soprano, D.R. and Soprano, K.J. (1988) Evidence that the time of entry into S is determined by events occurring in early G1. *J. Biol. Chem.*, **263**, 12751–12758.

Cowan, N.J., Dobner, P.R., Fuchs, E.V. and Cleveland, D.W. (1983) Expression of human alpha-tubulin genes: interspecies conservation of 3' untranslated regions. *Mol. Cell. Biol.*, **3**, 1738–1745.

Dingwall, C. and Laskey, R.A. (1991) Nuclear targeting sequences—a consensus? *Trends Biol.*, **16**, 478–481.

Eilers, M., Picard, D., Yamamoto, K. and Bishop, J.M. (1989) Chimaeras between the Myc oncoprotein and steroid receptors cause hormone dependent transformation of cells. *Nature*, **340**, 66–68.

Eilers, M., Schirm, S. and Bishop, J.M. (1991) The Myc protein activates transcription of the alpha-prothymosin gene. *EMBO J.*, **10**, 133–141.

Eisenman, R.N., Tachibana, C.Y., Abrams, H.D. and Hann, S.R. (1985) V-myc and c-myc encoded proteins are associated with the nuclear matrix. *Mol. Cell. Biol.*, **5**, 114–126.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.J. and Hancock, D.C. (1992) Induction of apoptosis in fibroblasts by c-Myc protein. *Cell*, **69**, 119–128.

Ferré-D'Amare, A.R., Prendergast, G.C., Ziff, E.B. and Burley, S.K. (1993) Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. *Nature*, **363**, 38–44.

Fisher, F. and Goding, R.C. (1992) Single amino acid substitutions alter helix-loop-helix protein specificity for bases flanking the core CANNTG motif. *EMBO J.*, **11**, 4103–4109.

Ford, M.J., Lane, J.A. and Lane, D.P. (1988) Nuclear protein with sequence homology to translation initiation factor IF-4A. *Nature*, **332**, 736–738.

- Gaubatz, S., Meichle, A. and Eilers, M. (1994) An E-box element localized in the first intron mediates regulation of the prothymosin  $\alpha$  gene by *c-myc*. *Mol. Cell. Biol.*, **14**, 3853–3862.
- Gaubatz, S., Imhof, A., Dorsch, R., Werner, O., Mitchell, P., Buettner, R. and Eilers, M. (1995) Transcriptional activation by Myc is under negative control by the transcription factor AP-2. *EMBO J.*, **14**, 1508–1519.
- Gregor, P.D., Sawadogo, M. and Roeder, R.G. (1990) The adenovirus major late transcription factor USF is a member of the helix–loop–helix group of regulatory proteins and binds DNA as a dimer. *Genes Dev.*, **4**, 1730–1740.
- Godbout, R. and Squire, J. (1993) Amplification of a DEAD box protein gene in retinoblastoma cell lines. *Proc. Natl Acad. Sci. USA*, **90**, 7578–7582.
- Gould, A.P., Brookman, J.J., Strutt, D.I. and White, R.A.H. (1990) Targets of homeotic gene control in *Drosophila*. *Nature*, **348**, 308–311.
- Gu, W., Cechova, K., Tassi, V. and Dalla-Favera, R. (1993) Opposite regulation of gene transcription and cell proliferation by *c-Myc* and Max. *Proc. Natl Acad. Sci. USA*, **90**, 2935–2939.
- Halazonetis, T.D. and Kandil, A.N. (1991) Determination of the *c-Myc* DNA-binding site. *Proc. Natl Acad. Sci. USA*, **88**, 6162–6166.
- Hann, S.R., King, M.W., Bentley, D.L., Anderson, C.W. and Eisenman, R.N. (1988) A non-AUG translational initiation in *c-myc* exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymphoma. *Cell*, **52**, 185–195.
- Henikoff, S. (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene*, **28**, 351–359.
- Henikoff, S. and Eghtedarzadeh, M.K. (1987) Conserved arrangement of nested genes in the *Drosophila* Gart locus. *Genetics*, **117**, 711–725.
- Hewish, D.R. and Burgoyne, L.A. (1973) Chromatin sub-structure. The digestion of chromatin DNA at regularly spaced sites by a nuclear deoxyribonuclease. *Biochem. Biophys. Res. Commun.*, **52**, 504–510.
- Hickstein, D.P., Black, A.L. and Collins, S.J. (1988) Regulation of expression of the CD11b and CD18 subunits of the neutrophil adherence receptor during human myeloid differentiation. *J. Biol. Chem.*, **264**, 21812–21817.
- Hümberlin, M., Safer, B., Chiorini, J.A., Hershey, J.W.B. and Cohen, R.B. (1989) Isolation and characterization of the promoter and flanking regions of the gene encoding the human protein-synthesis-initiation factor 2 $\alpha$ . *Gene*, **81**, 315–324.
- Hurlin, P., Ayer, D.E., Grandori, C. and Eisenman, R.N. (1994) The Max transcription factor network: involvement of Mad in differentiation and an approach to the identification of target genes. *Cold Spring Harbor Symp. Quant. Biol.*, **59**, 109–116.
- Hurlin, P.J., Quéva, C., Koskinen, P.J., Steingrímsson, E., Ayer, D.E., Copeland, N.G., Jenkins, N.A. and Eisenman, R.N. (1995) Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress *c-Myc*-dependent transformation and are expressed during neural and epidermal differentiation. *EMBO J.*, **14**, 5646–5659.
- Inghirami, G., Grignani, F., Sternas, L., Lombardi, L., Knowles, D.M. and Dalla-Favera, R. (1990) Down-regulation of LFA-1 adhesion receptors by *c-myc* oncogene in human B lymphoblastoid cells. *Science*, **250**, 682–686.
- Iost, I. and Dreyfus, M. (1994) mRNAs can be stabilized by DEAD box proteins. *Nature*, **372**, 193–196.
- Kahana, C. and Nathans, D. (1984) Isolation of cloned cDNA encoding mammalian ornithine decarboxylase. *Proc. Natl Acad. Sci. USA*, **81**, 3645–3649.
- Kato, G., Barrett, J., Villa-Garcia, M. and Dang, C.V. (1990) An aminoterminal *c-Myc* domain required for neoplastic transformation. *Mol. Cell. Biol.*, **10**, 5914–5920.
- Kozak, M. (1986) Point mutations define a sequence around the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell*, **44**, 283–292.
- Kretzner, L., Blackwood, E.M. and Eisenman, R.N. (1992) The Myc and Max proteins possess distinct transcriptional activities. *Nature*, **359**, 426–429.
- Larsson, L.-G., Pettersson, M., Öberg, F., Nilsson, K. and Luscher, B. (1994) Expression of *mad*, *mx1*, *max*, and *c-myc* during induced differentiation of hematopoietic cells: opposite regulation of *mad* and *c-myc*. *Oncogene*, **9**, 1247–1252.
- Lazaris-Karatzas, A., Montine, K.S. and Sonenberg, N. (1990) Malignant transformation by eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature*, **345**, 544–547.
- Lasko, P.F. and Ashburner, M. (1988) The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A. *Nature*, **335**, 611–617.
- Li, L., Nerlov, C., Prendergast, G., MacGregor, D. and Ziff, E.B. (1994) *C-Myc* represses transcription *in vivo* by a novel mechanism dependent on the initiator element and Myc box II. *EMBO J.*, **13**, 4070–4079.
- Linder, P., Lasko, P.F., Ashburner, M., Leroy, P., Nielsen, P.J., Nishi, K., Schnier, J. and Slonimski, P.P. (1989) Birth of the D-E-A-D box. *Nature*, **337**, 121–122.
- Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G. and Evan, G.I. (1995) A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res.*, **23**, 1686–1690.
- Lombardi, L., Newcomb, E.W. and Dalla-Favera, R. (1987) Pathogenesis of Burkitt lymphoma: expression of an activated *c-myc* oncogene causes tumorigenic conversion of EBV-infected human B lymphoblasts. *Cell*, **49**, 161–170.
- Lu, D. and Yunis, J.J. (1992) Cloning, expression and localization of an RNA helicase gene from a human lymphoid cell line with chromosomal breakpoint 11q23.3. *Nucleic Acids Res.*, **20**, 1967–1972.
- Manohar, C.F., Salwen, H.R., Brodeur, G.M. and Cohn, S.L. (1995) Co-amplification and concomitant high levels of expression of a DEAD box gene with MYCN in human neuroblastoma. *Genes, Chromosomes Cancer*, **14**, 196–203.
- Meichle, A., Philipp, A. and Eilers, M. (1992) The functions of Myc proteins. *Biochim. Biophys. Acta*, **1114**, 129–146.
- Nielsen, P.J. and Trachsel, H. (1988) The mouse protein synthesis initiation factor 4A gene family includes two related functional genes which are differentially expressed. *EMBO J.*, **7**, 2097–2105.
- Niwa, Q., Kumazaki, T., Tsukiyama, T., Soma, G., Miyajima, N. and Yokoro, K. (1990) A cDNA clone overexpressed and amplified in a mouse teratocarcinoma line. *Nucleic Acids Res.*, **18**, 6709.
- Nottenburg, C., Gallatin, W.M. and St. John, T. (1990) Lymphocyte HEV adhesion variants differ in the expression of multiple gene sequences. *Gene*, **95**, 279–284.
- Packham, G. and Cleveland, J. (1994) Ornithine decarboxylase is a mediator of *c-Myc*-induced apoptosis. *Mol. Cell. Biol.*, **14**, 5741–5747.
- Papoulas, O., Williams, N.G. and Kingston, R.E. (1992) DNA binding activities of *c-Myc* purified from eukaryotic cells. *J. Biol. Chem.*, **267**, 10470–10480.
- Pause, A., Methot, N. and Sonenberg, N. (1993) The HRIGRXXR region of the DEAD box RNA helicase eukaryotic translation initiation factor 4A is required for RNA binding and ATP hydrolysis. *Mol. Cell. Biol.*, **13**, 6789–6798.
- Penn, L.J., Brooks, M.W., Laufer, E.M., Littlewood, T.D., Morgenstern, J.P., Evan, G.I., Lee, W.M.F. and Land, H. (1990) Domains of human *c-myc* protein required for autosuppression and cooperation with ras are overlapping. *Mol. Cell. Biol.*, **10**, 4961–4966.
- Philipp, A., Schneider, A., Vasrick, I., Finke, K., Xiong, Y., Beach, D., Altalio, K. and Eilers, M. (1994) Repression of cyclin D1: a novel function of Myc. *Mol. Cell. Biol.*, **14**, 4032–4043.
- Prendergast, G.C. and Cole, M.D. (1989) Posttranscriptional regulation of cellular gene expression by the *c-myc* oncogene. *Mol. Cell. Biol.*, **9**, 124–134.
- Prendergast, G.C. and Ziff, E.B. (1991) Methylation-sensitive sequence-specific DNA binding by the *c-Myc* basic region. *Science*, **251**, 186–189.
- Prendergast, G.C., Lawe, D. and Ziff, E.B. (1991) Association of Myn, the murine homologue of Max, with *c-Myc* stimulates methylation-sensitive DNA-binding and Ras cotransformation. *Cell*, **65**, 395–407.
- Reisman, D., Elkind, N.B., Roy, B., Beamon, J. and Rotter, V. (1993) *C-Myc* trans-activates the p53 promoter through a required downstream CACGTG motif. *Cell Growth Differ.*, **4**, 57–65.
- Rosenwald, I.B., Lazaris-Karatzas, A., Sonenberg, N. and Schmidt, E.V. (1993a) Elevated expression of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E. *Mol. Cell. Biol.*, **13**, 7358–7363.
- Rosenwald, I.B., Rhoads, D.B., Callanan, L.D., Isselbacher, K.J. and Schmidt, E.V. (1993b) Increased expression of eukaryotic translation initiation factors eIF-4E and eIF-2a in response to growth induction by *c-myc*. *Proc. Natl Acad. Sci. USA*, **90**, 6175–6178.
- Schmid, S.R. and Linder, P. (1992) DEAD protein family of putative RNA helicases. *Mol. Microbiol.*, **6**, 283–292.
- Solomon, D.L.C., Amati, B. and Land, H. (1993) Distinct DNA binding preferences for the *c-Myc*/Max and Max–Max dimers. *Nucleic Acids Res.*, **21**, 5372–5376.
- Solomon, D.L.C., Philipp, A., Land, H. and Eilers, M. (1995) Expression of cyclin D1 mRNA is not upregulated by Myc in rat fibroblasts. *Oncogene*, **11**, 1893–1897.

- Squire, J.A., Thorner, P.S., Weitzman, S., Maggi, J.D., Dirks, P., Doyle, J., Hale, M. and Godbout, R. (1995) Co-amplification of MYCN and a DEAD box gene (DDX1) in primary neuroblastoma. *Oncogene*, **10**, 1417-1422.
- Stone, J., de Lange, T., Ramsay, G., Jacobovits, E., Bishop, J.M., Varmus, H. and Lee, W. (1987) Definition of human c-mycs that are involved in transformation and nuclear localization. *Mol. Cell. Biol.*, **7**, 1697-1709.
- Tikhonenko, A.T., Hartman, A.-R. and Linial, M.L. (1993) Overproduction of v-Myc in the nucleus and its excess are not required for avian fibroblasts transformation. *Mol. Cell. Biol.*, **13**, 3623-3631.
- Tobias, K.E., Shor, J. and Kahana, C. (1995) C-Myc and Max transregulate the mouse ornithine decarboxylase promoter through interaction with two downstream CACGTG motifs. *Oncogene*, **11**, 1721-1727.
- Tokunaga, K., Nakamura, Y., Sakata, K., Fujimori, K., Okubo, M., Sawada, K. and Sakiyama, S. (1987) Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. *Cancer Res.*, **47**, 5616-5619.
- Wagner, A.J., Meyers, C., Laimins, L.A. and Hay, N. (1993) C-Myc induces the expression and activities of ornithine decarboxylase. *Cell Growth Differ.*, **4**, 879-883.
- Walter, J., Dever, C.A. and Biggin, M.D. (1994) Two homeo domain proteins bind with similar specificity to a wide range of DNA sites in *Drosophila* embryos. *Genes Dev.*, **8**, 1678-1692.
- Wassarman, D.A. and Steitz, J.A. (1991) Alive with DEAD proteins. *Nature*, **349**, 463-464.
- Weintraub, H., Genetta, T. and Kadash, T. (1994) Tissue-specific gene activation by MyoD: determination of specificity by cis-acting repression elements. *Genes Dev.*, **8**, 2203-2211.
- Wessel, D. and Flugge, U.I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.*, **138**, 141-143.
- Westin, E.H., Gallo, R.C., Arya, S.K., Eva, A., Souza, L.M., Baluda, M.A., Aaronson, S.A. and Wong-Staal, F. (1982) Differential expression of the *amv* gene in human hematopoietic cells. *Proc. Natl Acad. Sci. USA*, **79**, 2194-2198.
- Wu, C. (1989) Analysis of hypersensitive sites in chromatin. *Methods Enzymol.*, **170**, 269-289.

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