

The DNA replication-related element-binding factor (DREF) is a transcriptional regulator of the *Drosophila myb* gene

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Abstract

Drosophila melanogaster possesses a single gene, *Dm myb*, that is closely related to the vertebrate proto-oncogene *c-Myb*, and its other family members (*A-Myb* and *B-Myb*), all of which encode transcription factors. *Dm myb* is expressed in all proliferating cells throughout development, and previous studies demonstrate that *Dm myb* promotes both S-phase and M-phase in proliferating cells, while preserving diploidy by suppressing endoreduplication. We have initiated a characterization of the mechanisms that regulate *Dm myb* expression, and we report here that the transcriptional activator DREF (the DNA replication-related element binding factor) activates *Dm myb* transcription via two binding sites located in the 5' flanking region; that the *Dm myb* promoter lacks a prototypical TATA box sequence and instead appears to use an initiator/downstream promoter element (Inr/DPE) type promoter; and that *Dm myb* expression is regulated at the translational as well as transcriptional level. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The proto-oncogene *c-Myb* (*Myb* – Human Gene Nomenclature Database) is the cellular homolog of the transduced retroviral oncogene *v-myb*, which induces myeloid leukemia in chickens and transforms myeloid cells in culture. Mutations affecting *c-Myb* have been implicated in the genesis of neoplastic disease in mice and humans (Oh and Reddy, 1999). *c-Myb* is now known to represent a small gene family in vertebrates which has two other closely related members, *A-Myb* and *B-Myb* (*Myb11* and *Myb12*, respectively – Human Gene Nomenclature Database) (Nomura et al., 1988). These three genes, which constitute the *Myb* gene family, encode nuclear, sequence specific DNA-binding proteins that can regulate transcription, and have been implicated in regulatory decisions affecting cell

proliferation, differentiation and apoptosis (for a comprehensive review, see Oh and Reddy, 1999).

A-Myb and *c-Myb* are both expressed in tissue-specific patterns during development and in adults, whereas *B-Myb* appears to be ubiquitously expressed in proliferating tissues (Oh and Reddy, 1999). Several lines of evidence indicate that vertebrate *Myb* genes are involved in regulating cell proliferation, particularly at the G₁/S transition and for progression through S-phase (Oh and Reddy, 1999): mRNA levels of all three vertebrate *Myb* genes fluctuate in a cell-cycle dependent manner in a subset of cell types; the cell cycle profiles are distinct, but the transcript levels of each *Myb* gene begin to rise at some point during G₁ and reach peak levels at a later stage of the cell cycle; and in general, within the tissues in which the individual *Myb* genes are expressed, there is a direct correlation between expression levels and the proliferative capacity of the cell type. The E2F transcription factor, which promotes S-phase, has been implicated in the regulation of *B-Myb* and *c-Myb* expression (Sala et al., 1994; Oh and Reddy, 1999; Humbert et al., 2000). In some, but not all types of cultured cells, altering the levels of *Myb* expression has profound consequences, with abnormally high levels promoting proliferation and interfering with differentiation, and reduced levels of *Myb* blocking proliferation (Oh and Reddy, 1999). In mice, knockout mutations of *A-Myb* or *c-Myb* lead to

Abbreviations: bp, base pairs; kbp, kilobase pairs; mRNA, messenger RNA; cDNA, DNA complementary to RNA; UTR, untranslated region; *Dm myb*, *Drosophila myb* gene; DMyb, *Drosophila Myb* protein; DRE, DNA replication-related element; DREF, DNA replication-related element binding factor; Inr, initiator; DPE, downstream promoter element; IRES, internal ribosome entry site; GST, glutathione-S-transferase; PCNA, proliferating cell nuclear antigen

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defects in proliferation of specific cell types (Oh and Reddy, 1999). A knockout of B-Myb, however, results in very early embryonic lethality, indicating that its ubiquitous expression pattern may reflect a universal role in cell proliferation (Tanaka et al., 1999).

Drosophila melanogaster possesses a single gene, *Dm myb*, that is closely related to the vertebrate *Myb* gene family (Katzen et al., 1985; Peters et al., 1987). The protein encoded by *Dm myb* (DMyb) shares four regions of homology with vertebrate Myb proteins and is equally related to A-Myb, B-Myb, and c-Myb (Katzen et al., 1998). We have demonstrated that DMyb shares biochemical properties with the vertebrate Myb proteins. DMyb binds to a similar consensus sequence and activates transcription from a reporter construct regulated by vertebrate Myb proteins (Jackson et al., 2001). *Dm myb* is expressed in all proliferating tissues, but not at detectable levels in endoreduplicating cells (Katzen and Bishop, 1996). We have previously analysed the effects of both reducing and increasing the levels of *Dm myb* activity within the context of the developing animal and found that *Dm myb* plays multiple roles in cell cycle (Katzen et al., 1998; Fung et al., 2002; Fitzpatrick, C.A., 2002). In diploid cells, DMyb promotes both S-phase and M-phase, and acts to preserve diploidy by suppressing endoreduplication. Mitotic defects observed in the loss-of-function mutant alleles of *Dm myb* indicate that it is required to maintain genomic integrity and may be involved in coordinating centrosomal and nuclear cell cycles.

We have initiated studies of the regulation of *Dm myb* expression, and report that the core promoter for *Dm myb* lacks a consensus TATA box sequence and instead, may be dependent on an initiator/downstream promoter element (Inr/DPE). There is no E2F binding site upstream of *Dm myb*, but we found two perfect matches for the DNA replication-related element binding factor (DREF) located within 150 bp upstream of the start of the *Dm myb* transcript, a region that can direct gene expression from reporter constructs in transient transfection assays. DREF is known to regulate a number of *Drosophila* genes involved in DNA replication and/or cell cycle progression, including the *dE2F* gene (Hirose et al., 1996; Ohno et al., 1996; Takahashi et al., 1996; Yamaguchi et al., 1996; Sawado et al., 1998; Lefai et al., 2000; Ruiz De Mena et al., 2000). We show that DREF binds to the DRE sites upstream of *Dm myb*, and that the DRE sites are required for efficient promoter activity. Finally, we present evidence that *Dm myb* expression is regulated at the translational as well as the transcriptional level, and that translational regulation may be the more important of the two.

2. Materials and methods

2.1. Clones, plasmid constructions and sequencing

Dm myb genomic clones have been previously described

(Katzen et al., 1985). Fragments of *Dm myb* 5'-genomic fragment were subcloned into pBlueScript II SK(-) and sequenced using T3 and T7 primers with T7 Sequenase version 2.0 sequencing kit (USB, Cleveland, OH).

cDNA clones were isolated from a library prepared from embryonic polyadenylated RNA (Poole et al., 1985). All *Dm myb* promoter constructs described in this study were derived from the pKB vector, which was generated by cloning the *KpnI/BamHI* fragment containing the multiple cloning site (MCS), firefly luciferase cDNA and a polyadenylation site from the pGL3B vector (Promega) into pBlueScript II SK(-) (Stratagene) digested with *KpnI* and *BamHI*. (Note: pGL3B could not be used because it contained two DRE sites upstream of the polylinker.) To begin a dissection of the *Dm myb* promoter, the following restriction enzyme fragments were cloned into the MCS of pKB (where +1 corresponds to the start of the *Dm myb* transcript): -1058 to +72 *EcoRI/BclII*, -699 to +72 *BglIII/BclII*, -152 to +72 *SspI/BclII*, and -51 to +72 *ClaI/BclII*. Note that the *EcoRI* site referred to here is present in some strains (e.g. Canton S), but not others (e.g. Oregon R) (Katzen et al., 1985), and is not present in the *D. melanogaster* genomic scaffold sequence that displays the complement of *Dm myb* (GenBank accession no. AE003500).

Mutations in DRE sites were created by partial digestion with *ClaI*, followed by generation of blunt ends with Klenow DNA polymerase and self-ligation of the resulting plasmid. This procedure creates a 2 bp insertion converting consensus DREF site 5'-TATCGATA-3' into 5'-TATCGC-GATA-3', a change that has been shown to abolish DREF binding and to lead to extensive reductions in the activities of DRE-dependent promoters (Hirose et al., 1993; Ohno et al., 1996). Clones containing mutations in individual DRE sites were selected and subjected to the second round of mutagenesis by *ClaI* and Klenow polymerase. All mutations were confirmed by sequencing. To investigate the possibility that a DPE is important for *Dm myb* expression, a *SspI/ScaI* fragment (-152 to +9) was cloned into pKB. Inspection of the +28 to +33 region contributed by the vector in this construct showed a poor match to the DPE consensus.

2.2. Oligonucleotides

Double-stranded 30 bp oligonucleotides containing 24 bp DRE sequences and the 6 bp linker sequences were synthesized by Integrated DNA Technologies, Coralville, IA. The DRE sequences correspond to *Dm myb* DRE1, *Dm myb* DRE2 and a mutated version of DRE in the context of *Dm myb* DRE1, respectively.

DRE1: 5'-GATCTAGCTAAATATCGATACTATCCCA-3'
3'-ATCGATTTATAGCTATGATAGGGTCTAG-5'

DRE2: 5'-GATCTGATTATTTATCGATAGATTTTAA-3'
3'-ACTAATAAATAGCTATCTAAAATTCTAG-5'

DRE-M: 5'-GATCTAGCTAAATTTCCAAACTATCCCA-3'
3'-ATCGATTTAAAGGTTTGATAGGGTCTAG-5'

2.3. Electrophoretic mobility shift assays (EMSA)

For EMSA experiments, double-stranded ^{32}P -labeled DRE1, DRE2, or DRE-M oligonucleotides (50 pg, 10^4 cpm) were incubated for 10 min on ice with 1 $\mu\text{g}/\mu\text{l}$ poly(dI-dC) (non-specific competitor) and either 50 ng of purified bacterially-expressed GST-DREF(1–125) fusion protein, or with 5 μg Schneider cell nuclear extract protein in a 20 μl reaction mixture. The GST-DREF fusion protein includes the N-terminal 125 amino acids of DREF, a region previously shown to be sufficient for efficient dimerization and DNA-binding activities (Hirose et al., 1996). For competition experiments, unlabeled oligonucleotides were added to the reaction mixture before the radioactively labeled oligonucleotide. For the supershift analysis, 1 μg of mouse monoclonal anti-DREF MAb4 (provided by A. Matsukage; Hirose et al., 1996) was added to the binding reaction. Samples were electrophoresed on 5% polyacrylamide gels.

Nuclear extracts were prepared from *Drosophila* Schneider cells (SL2; Cherbas and Cherbas, 2000) as previously described (Jackson et al., 2001). The GST-DREF (1–125) fusion protein was expressed from a pGEX-DREF(1–125) clone generously provided by A. Matsukage (Hirose et al., 1996) in *Escherichia coli* BL21 (Pharmacia), and purified as previously described (Jackson and Kaminski, 1995).

2.4. Transient transfection assays

Drosophila Schneider cells (S2) were cultured at 25 °C in Schneider's *Drosophila* medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan UT) and penicillin/streptomycin (Life Technologies). Schneider cells were plated at 5×10^6 cells per P60 dish 24 h before DNA transfection using the calcium phosphate co-precipitation method (Cherbas and Cherbas, 2000). Each transfection included 5 μg of the pKB plasmid containing a *Dm myb* promoter fragment inserted upstream of the firefly (*Photinus pyralis*) luciferase. For normalization, all transfections were cotransfected with 1 μg of pRL-CMV plasmid (Promega; Madison, WI) which expresses the *Renilla reniformis* luciferase. Cells were harvested 48 h after transfection and processed using the Dual-Luciferase Reporter Assay System by Promega. Firefly and *Renilla* luciferase luminescence values were determined using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). All transient transfection data reported in this paper represent the means from three independent experiments, each performed in triplicate.

2.5. Analysis of effects of 5'-UTR on translation efficiency

DNA fragments containing either the full-length *Dm myb* cDNA or a *Dm myb* cDNA in which 500 bases, including all upstream AUGs, were removed from the 5'-UTR using a *Bgl*II site (107 bases upstream of the DMyb AUG), were cloned into pBlueScript II SK(-) (Stratagene) for in vitro

transcription/translation. [^{35}S]Methionine-labeled proteins were produced using the TNT coupled reticulocyte lysate system (Promega) and fractionated on 12.5% SDS-polyacrylamide gels.

For expression in cells that do not contain endogenous DMyb protein, the same cDNA fragments were cloned into the murine retroviral pLNCX vector for expression in the human embryonic kidney cell line, 293T. Use of this vector and cell line, as well as immunoblotting procedures and the rabbit polyclonal antisera raised against the C-terminal region of DMyb have been previously described (Jackson et al., 2001). Transfection efficiencies were monitored visually by cotransfection with pEGFP-N1 (Clontech), which expresses the enhanced green fluorescent protein gene via the CMV promoter.

3. Results

3.1. Clarification of the *Dm myb* transcript structure

The expression pattern and the genomic and cDNA sequences of the *Dm myb* gene have been previously described (Katzen et al., 1985; Peters et al., 1987; Katzen and Bishop, 1996; Adams et al., 2000), but differences between the published sequences exist and the promoter and enhancer elements required to execute appropriate expression have not been characterized. When the *Drosophila* genome annotation became available, the proposed positions of transcript initiation and termination differed from previously published data (Peters et al., 1987; Adams et al., 2000; and see Fig. 1). To resolve these discrepancies, we isolated more than 40 cDNA clones representing embryonic *Dm myb* mRNA. Sequencing of the 5' ends revealed that many of the cDNA clones extended to essentially the same position as that published by Peters et al. (1987) (GenBank accession no. X05939), and that none extended further. The position of the mRNA initiation site was also consistent with primer initiated reverse transcription data which revealed that the highest primer extension band co-migrated with the equivalent fragment from one of the putative full-length cDNA clones (data not shown). The only discrepancy in sequences was that ours did not include a 'G' as the first nt. Since there is not a corresponding 'G' in the genomic sequence, we conclude that it is likely to have been generated during the cloning process. Our results and those of Peters et al. (1987) establish that the actual point of initiation is 23 bases upstream of the position predicted by the genome project (nt 148,291 vs. 148,268 in *D. melanogaster* genomic scaffold, GenBank accession no. AE003500, which displays the complement of *Dm myb* – numbering below also refers to this accession number; also see Fig. 1).

The 3' ends of the cDNAs were heterogeneous and included representatives corresponding to both the published and predicted terminations (Peters et al., 1987;

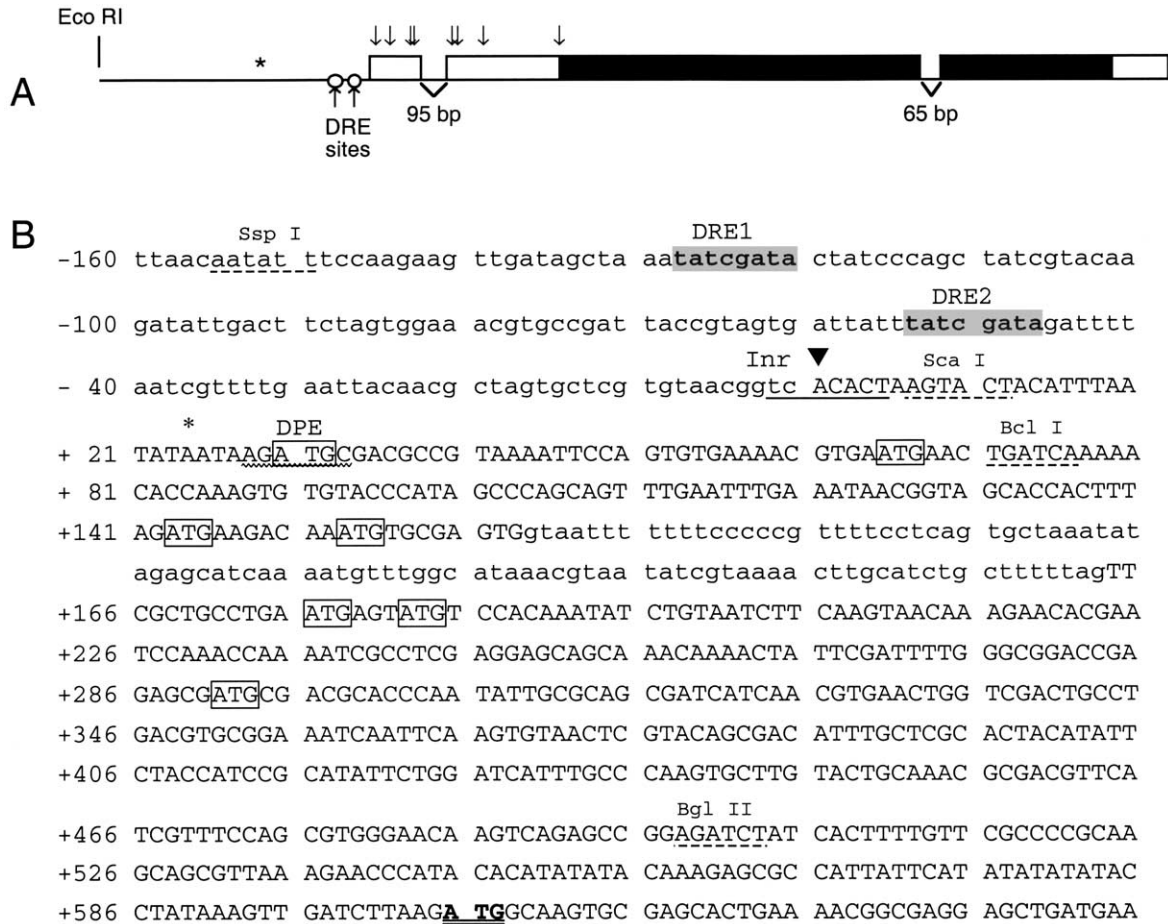


Fig. 1. Genomic structure of the *Dm myb* gene and sequences involved in its regulation. (A) Schematic representation of the *Dm myb* genomic region and 5'-flanking sequences addressed in these studies (5' to the left). Filled bars represent protein coding regions; non-filled bars represent non-coding regions of the mRNA. Positions of the two small introns are shown, including the newly identified 95 bp intron in the 5'-UTR. The seven AUG codons in the 5'-UTR that precede the DMyb coding sequence and the starting DMyb codon are indicated with arrows. Open circles in the 5'-flanking region represent the two consensus DRE sites. The asterisk indicates the 3'-end of coding sequence (located at -384 bp with respect to the start of the *Dm myb* transcript) for the previously unidentified *alkB* gene, which is located upstream of *Dm myb* and is transcribed in the same orientation. (B) Nucleotide sequence of the 5'-UTR and 5'-flanking region of the *Dm myb* gene. The nucleotides are numbered relative to *Dm myb* transcript, where the transcription start site (indicated by an arrowhead) is +1. Sequences in the 5'-flanking region and intron are in lower case; sequences in the transcript are in upper case. Sequences corresponding to putative initiator (Inr; TCA₊₁CACT) and downstream promoter (DPE; AG₊₂₉ATGC) elements are indicated and underlined with straight and wavy lines, respectively. The two DRE consensus sequences are highlighted in gray. The starting methionine codon is shown in bold and underlined with a double line, and the seven ATGs in the 5'-UTR that precede it are boxed. The asterisk indicates the transcript initiation site predicted by the *Drosophila* genome project (Adams et al., 2000; GenBank accession no. AE003500). Finally, restriction enzymes sites for *SspI*, *ScaI*, *BclI*, and *BglII*, which are used in these studies are indicated and underlined with dashed lines.

Adams et al., 2000). The heterogeneity is likely to result from the presence of two overlapping AAUAAA poly(A) addition signal sequences in the 3'-UTR (complement of nt 145,356–148,365) and an AAUCAA sequence downstream (complement of nt 145,339–148,344). Based on their positions, the latter sequence, which has been shown previously to function as a poly(A) addition signal for some transcripts (Edwalds-Gilbert et al., 1997), is likely to be the one that was used for the 3' termination reported by Peters et al. (1987).

Comparison of genomic and cDNA sequences confirmed the presence of an intron that disrupts coding sequence (complement of nt 146,214–146,277), but also revealed a 95 nt intron in the 5'-UTR (complement of nt 148,034–

148,128), which was not predicted by the annotated sequence (Fig. 1). The sequences of the splice junctions and adjacent nucleotides for both introns conform to the consensus sequences established for splice donor and acceptor sites (Mount, 1982). The presence of an intron in the 5'-UTR accounts for another discrepancy between the sequences published for the cDNA and predicted by the *Drosophila* genome project (Peters et al., 1987; Adams et al., 2000).

3.2. Initial characterization of upstream sequences required for the *Dm myb* expression

We have previously reported that a *Dm myb* transgene represented by a 6.7 kbp genomic fragment extending 2 kbp

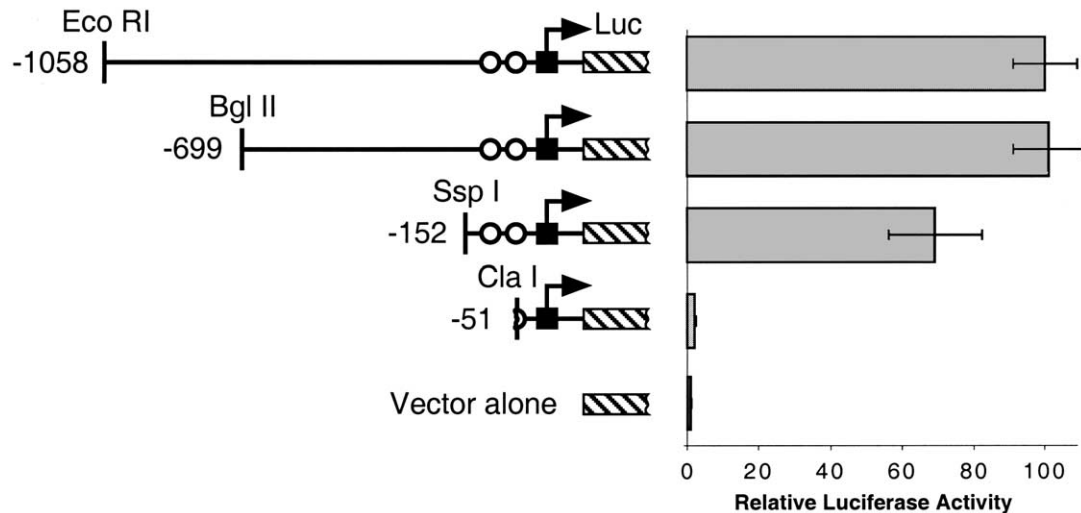


Fig. 2. Analysis of upstream regulatory region required for *Dm myb* expression in cultured cells. Four fragments that were cloned upstream of the luciferase reporter gene in the pKB vector and used for initial analysis of *Dm myb* promoter activity in cultured *Drosophila* Schneider cells are schematically represented on the left. Restriction enzymes sites used to generate the 5'-ends and their positions relative to the start of *Dm myb* transcription are indicated. The 3'-end of the fragments was the same for all constructs, and was located at position +72 (*Bcl*I site). Open circles represent DRE consensus sites (TATCGATA). In this figure and in Figs. 4 and 5, the *Dm myb* transcription start site in each construct is represented by a black box with an arrow, and hatched boxes represent the firefly luciferase cDNA (Luc). The relative promoter activity, as measured by luciferase activity, is graphically represented to the right of the corresponding construct. Luciferase values are represented relative to the longest construct (–1058 to +72), which was assigned a value of 100% (value for vector alone $\ll 1\%$). Transfections were performed as described in Section 2. Standard deviations are indicated.

upstream of the start of the *Dm myb* transcript is capable of completely rescuing all aspects of the mutant phenotype in two loss-of-function alleles of *Dm myb* (Katzen and Bishop, 1996; Katzen et al., 1998), indicating that this fragment is likely to contain the *cis*-regulatory sequences required for appropriate expression of *Dm myb* during development. These 5' flanking sequences were subcloned and sequenced prior to the release of the *D. melanogaster* genomic sequence (Adams et al., 2000), which was fortuitous since our analysis revealed the presence of a neighboring gene, *alkB*, which was not identified by the genome project because of a base-pair change that introduced a non-sense mutation in the coding region (Sharkov, N.V., Katzen, A.L., in preparation). *alkB* is transcribed in the same orientation as *Dm myb*, and the *alkB* coding sequence ends 384 bp upstream of the *Dm myb* transcript, indicating that the *cis*-regulatory sequences required to regulate *Dm myb* transcription are likely to be contained within this intergenic region.

Since E2F/DP transcription factors have been implicated in the regulation of vertebrate *Myb* genes, and *Dm myb* is expressed in all proliferating cells, we searched the upstream sequences for consensus E2F binding sites (*Drosophila* E2F has been shown to bind the same site as determined for mammals; Nevins, 1992; Dynlacht et al., 1994). No perfect matches were found in the 2 kbp upstream of the *Dm myb* transcript, although there were four partial matches with two mismatches each, all four of which were located within the *alkB* coding region. In transient transfection assays to monitor *Dm myb* promoter activity, the presence or absence of the most proximal of these partial matches (nt –963 to –956 with respect to transcription

initiation) had no effect on promoter activity (Fig. 2), indicating that it is unlikely that *Dm myb* is transcriptionally regulated by E2F, at least directly.

In *Drosophila*, the DREF transcription factor was originally thought to act in concert with E2F to specifically activate expression of DNA replication-related genes such as those encoding subunits of DNA polymerase α and PCNA (proliferating cell nuclear antigen) (Hirose et al., 1993, 1996; Takahashi et al., 1996; Yamaguchi et al., 1996; Huikeshoven and Cotterill, 1999). However, DREF has since been shown to be a positive regulator of a variety of genes involved in cell proliferation, including *cyclin A* and the *Drosophila* E2F gene (*dE2F*) itself (Ohno et al., 1996; Sawado et al., 1998), many of which have been shown to be targets of E2F regulation in mammals (Lee et al., 1995; Humbert et al., 2000; Izumi et al., 2000). In addition, DREF has recently been implicated in the regulation of several genes required for mitochondrial DNA replication, indicating that DREF may coordinate the regulation of nuclear and mitochondrial DNA replication (Lefai et al., 2000; Ruiz De Mena et al., 2000). Therefore, we hypothesized that *Dm myb* might also be regulated by DREF. A search of the 5' flanking sequences of *Dm myb* revealed two perfect matches to the 8 bp palindromic sequence 5'-TATCGATA, which represents the DNA replication-related element (DRE) that has been shown to be recognized by DREF. These sites were located at positions –128 to –121 (DRE1) and –54 to –47 (DRE2) with respect to the transcriptional initiation site (Fig. 1).

In initial studies to investigate the potential significance of the DRE sites for *Dm myb* expression, 5' flanking

segments of DNA that varied at the 5' end, but which all extended to +72 at the 3' end (where +1 corresponds to the start of the *Dm myb* transcript), were cloned into the pKB vector (see Section 2.1) upstream of a luciferase reporter gene. In transient transfection assays performed in *Drosophila* Schneider cells, little difference in promoter activity was observed between a construct extending to –152, which contains both DRE sites, and constructs extending much further 5', to –699 or –1058 (Fig. 2). In contrast, promoter activity was completely abolished when both DRE sites were deleted (–51 to +72), indicating that DREF might be a key regulator of *Dm myb* transcription.

3.3. DREF binds to the DRE sequences in the *Dm myb* promoter

To investigate DREF binding to the DRE sites upstream of the *Dm myb* transcription unit, gel electrophoretic mobility shifts assays (EMSA) were performed using radioactively labeled double-stranded oligonucleotides corresponding to sequences encompassing either DRE1 or DRE2 and adjacent nucleotides (Fig. 3). Both probes were efficiently bound and shifted by a bacterially expressed GST-DREF fusion protein. The specificity of these interactions was demonstrated by the ability of excess amounts of the unlabeled DRE1 or DRE2 oligonucleotides, but not an oligonucleotide in which the DRE site had been mutated (DRE-M), to interfere with the formation of GST-DREF/³²P-labeled DRE oligonucleotide complexes (Fig. 3A,B). In competition experiments, a 200-fold excess of unlabeled DRE2 oligonucleotide completely abolished complex formation between GST-DREF and either ³²P-DRE1 or ³²P-DRE2 but a 200-fold excess of unlabeled DRE1 oligonucleotide only partially diminished complex formation between GST-DREF and ³²P-DRE2. These results indicate that GST-DREF has a higher affinity for the DRE2 site than for the DRE1 site, but when DNase I footprinting analysis was performed with the GST-DREF protein, both DRE sites were protected (data not shown). Since recombinant DREF protein was used in this analysis, it did not address the presence of other regulatory sites in the promoter and enhancer regions, and it seems likely that additional protected sites will be revealed when nuclear extracts are used in footprinting reactions (see Section 4).

When radioactively labeled DRE1 and DRE2 probes were incubated with nuclear extracts prepared from Schneider cells, specific DNA-protein complexes were detected, which could be competed out with excess amounts of unlabeled DRE oligonucleotides (Fig. 3C,D). To determine whether DREF is the protein responsible for forming the observed DNA-protein complexes observed with the Schneider cell nuclear extracts, an anti-DREF mouse monoclonal antibody (mAb 4; Ohno et al., 1996), which recognizes an epitope in the DREF protein that is located outside of the DNA-binding domain, was added to the binding reaction. The anti-DREF antibody efficiently 'supershifted' the

DNA-protein complexes (Fig. 3C,D), indicating that the DREF protein present in Schneider cells is the major nuclear protein capable of binding the *Dm myb* DRE sites in vitro.

3.4. The DRE2 site is important for the promoter activity of the *Dm myb* gene

To directly examine the importance of the DRE sites for *Dm myb* promoter activity, the activity of the wild-type *Dm myb* promoter (–152 to +72 fused to luciferase) was compared with the activity of the promoter when the DRE sites had been either individually (DREmut1 or DREmut2) or doubly mutated (DREmut1,2). Results from transient transfection assays in Schneider cells indicate that the functional importance of the two DRE sites in the *Dm myb* promoter is not equivalent (Fig. 4A): the mutation in DRE1 did not significantly reduce the activity of the *Dm myb* promoter, whereas the mutation in DRE2 reduced promoter activity by approximately 50%. The DRE1 site does appear to contribute some functional activity, however, since promoter activity was further reduced in constructs bearing mutations in both DRE sites.

Interestingly, the functional significance of *Dm myb* DREs correlated with their relative affinities for GST-DREF. The *Dm myb* DRE2 site (but not the DRE1 site) has two flanking thymidylate residues two bp downstream from the core DRE site (5'-TATCGATA-gaTT), a feature previously reported to contribute to DRE function (Hirose et al., 1993). However, a later study showed that substitution of the two thymidylate residues in the DRE of the *PCNA* gene caused only modest reductions of promoter activity in transient transfection assays, leading the authors to conclude that the 8 bp core DRE sequence is essential and sufficient for DRE function (Yamaguchi et al., 1995). In addition, the dissimilarity in affinities of the two DRE sites was less apparent when Schneider cell nuclear extracts were used in the EMSAs (Fig. 3C,D) instead of GST-DREF. Therefore, the contrast in functional significance of the two DRE sites cannot be fully explained by differences in affinity, and is likely to also involve other contextual differences, such as the presence or absence of sites for other DNA-binding proteins.

3.5. The *Dm myb* core promoter lacks a prototypical TATA box

For TATA box-dependent promoters, an A/T rich sequence with the consensus 5'-TATAAA is typically located about 20–30 nucleotides upstream of the transcription start site. Although the 5' sequences upstream of the *Dm myb* transcript contain several AT rich regions (a common property of non-coding sequences in *Drosophila*), there are no consensus sequences for TATA box elements in the pertinent region (GenBank accession no. AE003500). The closest partial match (5'-TATTTA) is located more than 50 bp upstream (Fig. 1), and ideal matches don't occur until –225 and –247 with respect to the transcription

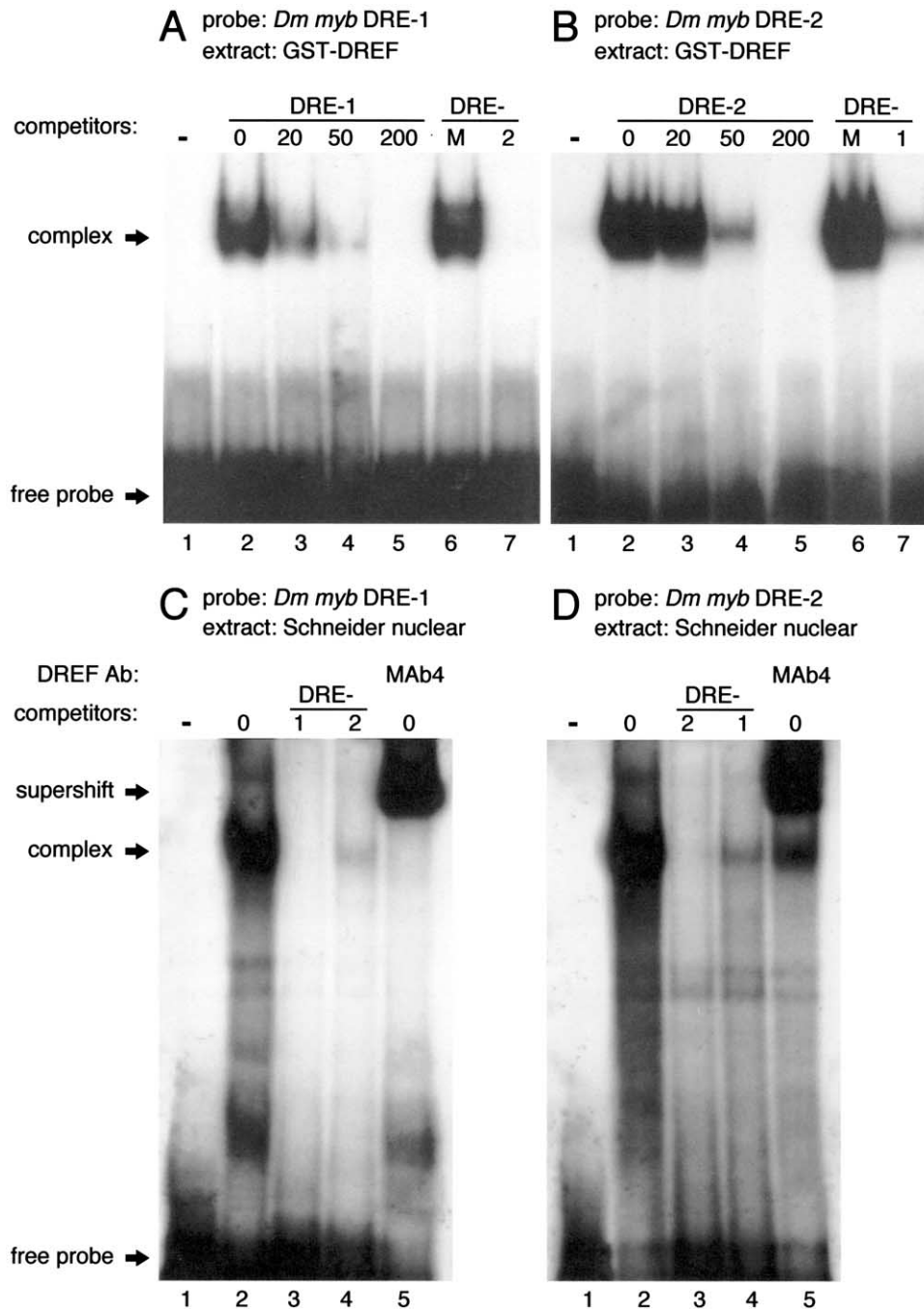


Fig. 3. DREF binds to the DRE sites in the *Dm myb* promoter. Electrophoretic mobility shift analysis was performed using radioactively labeled double-stranded oligonucleotides corresponding to sequences surrounding and including DRE1 (A,C) or DRE2 (B,D) of the *Dm myb* promoter and bacterially-expressed GST-DREF(1–125) fusion protein (A,B) or Schneider cell nuclear extract (C,D). Positions of bands corresponding to free probe, protein-DNA complexes, and ‘super-shifted’ complexes formed with anti-DREF antibodies are indicated on the left of panels A and C. (A) Lane 1, radiolabeled DRE1, free probe; lanes 2–7, radiolabeled DRE1 probe incubated with GST-DREF(1–125) with either no competitor (lane 2); 20-fold (lane 3), 50-fold (lane 4) or 200-fold (lane 5) molar excess of unlabeled DRE1 oligonucleotide; 200-fold molar excess of unlabeled DRE-M, an oligonucleotide with extensively mutated DRE core in the context of DRE1 of *Dm myb* (lane 6); or 200-fold molar excess of unlabeled DRE2 oligonucleotide (lane 7). (B) Lane 1, radiolabeled DRE2, free probe; lanes 2–7, radiolabeled DRE2 probe incubated with GST-DREF(1–125) with either no competitor (lane 2); 20-fold (lane 3), 50-fold (lane 4) or 200-fold (lane 5) molar excess of unlabeled DRE2 oligonucleotide; 200-fold molar excess of unlabeled DRE-M (lane 6); or 200-fold molar excess of unlabeled DRE1 oligonucleotide (lane 7). (C) Lane 1, radiolabeled DRE1, free probe; lanes 2–5, radiolabeled DRE1 probe incubated with 5 μ g of Schneider cell nuclear extract with either no competitor (lane 2); 100-fold of unlabeled DRE2 oligonucleotide (lane 3), 100-fold of unlabeled DRE1 oligonucleotide (lane 4) or 1 μ g of mouse monoclonal anti-DREF antibody MAb4 (lane 5). (D) Lane 1, radiolabeled DRE2, free probe; lanes 2–5, radiolabeled DRE2 probe incubated with 5 μ g of Schneider cell nuclear extract with either no competitor (lane 2); 100-fold molar excess of unlabeled DRE2 oligonucleotide (lane 3), 100-fold molar excess of unlabeled DRE1 oligonucleotide (lane 4) or 1 μ g of mouse monoclonal anti-DREF antibody MAb4 (lane 5).

initiation site (not shown). The latter sequences were not included in promoter constructs that were active in the transient transfection assays, indicating that they are not required for efficient promoter activity (see Section 3.2 and Fig. 2). Instead, sequence inspection indicates that core *Dm myb* promoter is likely to be dependent on a downstream promoter element (DPE; Fig. 1). In DPE-dependent promoters, the DPE functions cooperatively with an initiator (Inr) sequence at strictly maintained positions with respect to the transcriptional initiation site, to bind to TFIID and direct accurate and efficient initiation of transcription (Burke and Kadonaga, 1997). The sequence at the *Dm myb* transcription initiation site, 5'-TCA₊₁CACT (where A₊₁ is the transcription start site) matches the consensus sequence derived for the initiator (Inr) in mammals, 5'-Py-Py-A₊₁-N-T/A-Py-Py, and is a reasonably good fit with the Inr consensus sequence derived for *Drosophila*, 5'-T-C-A₊₁-G/T-T-T/C (Javahery et al., 1994; Purnell et

al., 1994). The downstream *Dm myb* sequences at the specified positions for the DPE, 5'-AG₊₂₉ATGC, is a 5 out of 6 match for the DPE consensus (5'-A/G-G₊₂₉-A/T-C/T-G-T), and falls within the functional range of sequences that have been shown to function as a DPE (Kutach and Kadonaga, 2000).

All of our initial constructs extended to the +72 position, which included the candidate Inr and DPE sequences. Therefore, to ascertain whether sequences downstream of the initiator site might be important for promoter activity, we generated a construct that started at position -152 and extended to +9, and compared its activity in transient transfection assays with the -152 to +72 construct used for our other studies (Fig. 4B). Luciferase activities for the -152 to +9 promoter construct were consistently lower than for the -152 to +72 construct, by about 50%. This is likely to be an underestimate of the decrease in promoter activity since deletion of the +9 to +72 region removes two AUG codons

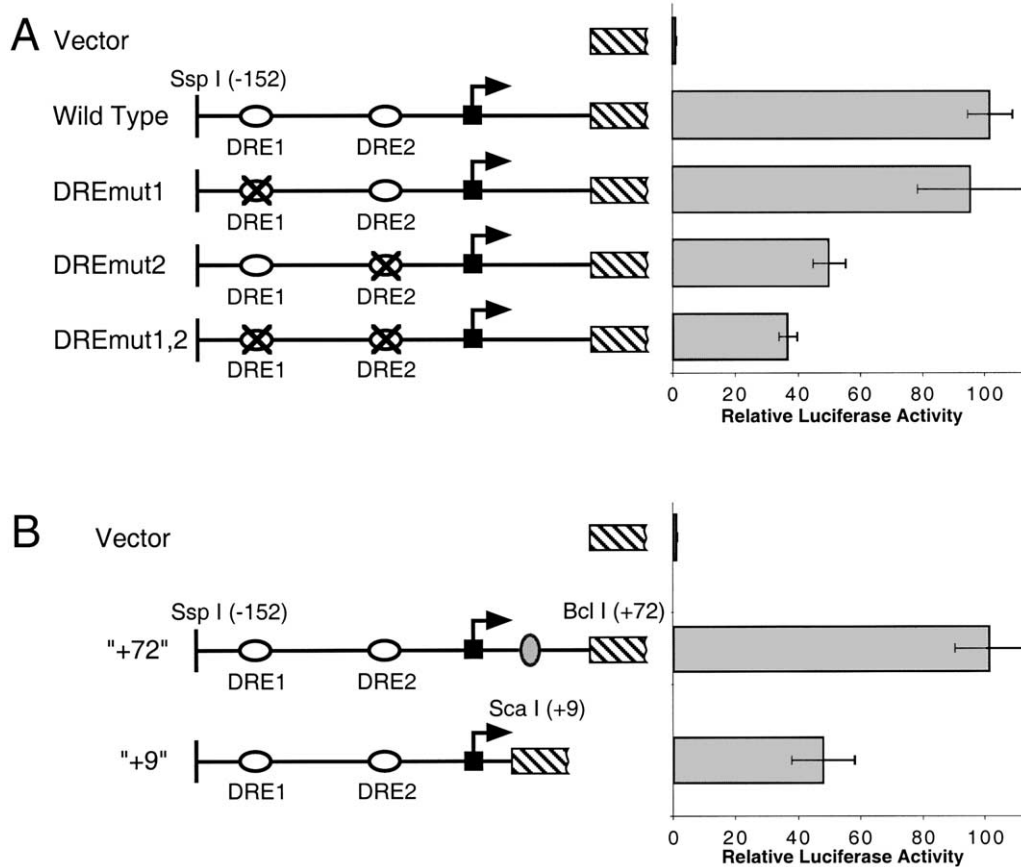


Fig. 4. Evaluation of the contribution made to *Dm myb* promoter activity by each DRE site and sequences downstream of the transcription initiation site. (A) Four *SspI/BclI* fragments (-152 to +72) representing the wild-type, singly mutated (DREmut1 or DREmut2), or doubly mutated (DREmut1,2) DRE sites (see Section 2.1) were cloned upstream of the luciferase reporter gene in the pKB vector, and are schematically represented on the left. Ovals represent the DRE sites; an X on top of the oval indicates a mutated site. (B) Two fragments sharing the same 5'-end (-152), but differing at the three ends were cloned upstream of the luciferase reporter gene in the pKB vector, and are schematically represented on the left. One construct contained a fragment extending to position +72 (*BclI*), which is the same position as all other constructs used in these studies; the other extended only to position +9 (*ScaI*) in order to remove the putative DPE. Open horizontal ovals represent the DRE sites; a solid vertical oval represents the putative DPE residing at positions +28 to +33. For both panels, the relative promoter activity, as measured by luciferase activity, is graphically represented to the right of the corresponding construct. Luciferase values are represented relative to the wild-type *SspI/BclI* (-152 to +72) construct, which was assigned a value of 100% (value for vector alone \ll 1%). Standard deviations are indicated.

that are located upstream of the starting codon for luciferase, which should increase the efficiency of luciferase translation. Therefore, this data indicates the +9 to +72 fragment contains important sequences for promoter function and is consistent with the *Dm myb* core promoter being DPE-dependent, although definitive conclusions cannot be drawn until site specific mutations are introduced into the putative Inr and DPE sequences.

3.6. *Dm myb* may be translationally as well as transcriptionally regulated

We have previously shown that *Dm myb* is expressed in all proliferating cells (Katzen and Bishop, 1996), and we present data here that DREF, a transcriptional activator of multiple genes involved in DNA replication and cell proliferation, activates *Dm myb*. However, in contrast to the vertebrate *Myb* genes (see Section 1), no cell cycle dependent fluctuation in *Dm myb* mRNA levels was observed, at least in embryos, and the levels of transcript decreased slowly in post-mitotic tissues (Katzen and Bishop, 1996). Preliminary in situ analysis of the DMyb protein in embryos indicates that the protein levels do not directly reflect the mRNA levels (Ramsay, G., unpublished results). These observations suggested that *Dm myb* might be subject to post-transcriptional regulation, and we provide evidence that at least one aspect of this regulation may be at the level of translational efficiency.

The *Dm myb* transcript has an unusually lengthy 5'-UTR with the AUG initiation codon at position +605 of the spliced transcript. In addition to its distance from the 5' end of the transcript, the AUG for the DMyb protein is the eighth AUG in the transcript (Fig. 1), a situation that is likely to lead to poor efficiency of translation since translation initiation sites

in eukaryotic mRNAs are usually reached via a scanning mechanism that begins at the 5' end (Kozak, 1999). According to consensus sequences derived by Kozak and others for the bases immediately surrounding AUGs that are required for efficient initiation of translation (Cavener, 1987; Kozak, 1999), none of the seven AUGs that precede the DMyb AUG are in an optimal context. However, that is also the case for the DMyb AUG, although it does display appropriate bases at the two positions that have been shown to be most critical for efficient initiation: an A at position -3 and a G at position +4 (where the A of the AUG is +1). None of the preceding seven AUGs have optimal bases (purines) at both of these positions and each is followed by an in-frame terminator codon that occurs before the start of the DMyb coding domain.

To examine the effects of the upstream AUGs on the translation of DMyb, in vitro transcription/translation experiments were performed on templates that either included the full-length 5'-UTR or that had been truncated to remove all upstream AUGs. As expected, the efficiency of translation was improved (two- to threefold) in the absence of the upstream AUGs (Fig. 5A). The effect was even more pronounced when a full-length *Dm myb* cDNA or one in which most of the 5'-UTR had been deleted to remove all upstream AUGs, were cloned into an expression vector and expressed in cultured cells. When Western blots containing equal amounts of protein were prepared from both cultures, approximately ten times more DMyb protein was detected in the cells expressing the truncated cDNA (Fig. 5B).

4. Discussion

We have investigated the extents and structure of the *Dm myb* transcriptional unit and found that it is composed of

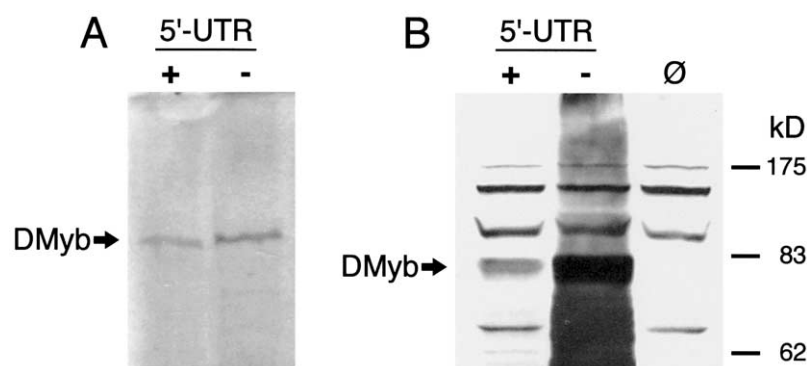


Fig. 5. Numerous upstream AUGs in the 5'-UTR inhibit translation. (A) In vitro translation of *Dm myb* mRNA is increased when all upstream AUGs are deleted. A full-length *Dm myb* cDNA clone containing seven upstream AUGs in the 5'-UTR (+) and a *Dm myb* cDNA clone in which all upstream AUGs were removed from the 5'-UTR (-) using a *Bgl*III site (see Fig. 1B for position) were subjected to in vitro transcription/translation using the TNT coupled reticulocyte lysate system from Promega. The resulting [³⁵S]methionine-labeled proteins were then fractionated on 12.5% SDS-polyacrylamide gels. Similar results were obtained with the wheat germ in vitro transcription/translation system (not shown). (B) Greater quantities of DMyb protein are produced in cultured cells when all upstream AUG codons are deleted. Nuclear fractions were prepared from 293T cells transfected with a pLNCX construct containing the full-length *Dm myb* cDNA (+), a pLNCX construct containing the *Dm myb* cDNA in which the 500 most 5' bases in the 5'-UTR had been removed (-); or pLNCX vector without insert (Ø). Transfection efficiencies monitored by EGFP fluorescence from cotransfected pEGFP-N1 were equivalent (26, 24, and 27%, respectively). Equal amounts of protein from these three samples were fractionated on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose. DMyb protein was detected using a rabbit polyclonal antisera raised against the C-terminal region of DMyb.

three exons and two small introns, one of which is in the 5'-UTR and had not been previously identified. Our results also indicate that the previously published cDNA sequence is complete and confirms the position of transcriptional initiation identified in their sequence (except for the first nucleotide), as opposed to the initiation site predicted by the *Drosophila* genome project (Peters et al., 1987; Adams et al., 2000). The identification of the actual site of initiation allowed us to determine that the *Dm myb* promoter does not contain a prototypical TATA box sequence, and is likely to be DPE-dependent. We also observed heterogeneity at the 3' end of the transcript due to the presence of multiple poly(A) addition signals. These results demonstrate the need for direct experimentation to test the validity of gene annotations from genome sequencing projects, which are based on computational prediction programs.

Our results demonstrate that the DREF/DRE system, previously shown to activate transcription of a wide array of DNA replication and cell cycle-related genes (Hirose et al., 1996; Ohno et al., 1996; Takahashi et al., 1996; Yamaguchi et al., 1996; Sawado et al., 1998; Lefai et al., 2000; Ruiz De Mena et al., 2000), plays an important role in regulation of *Dm myb* transcription. This correlates with our previous findings that *Dm myb* is expressed in all proliferating cells (Katzen and Bishop, 1996). However, two observations indicate that DREF is not the only transcription factor involved: i) mutations in both DRE sites lead to a significant reduction in *Dm myb* promoter activity, but not complete inactivation, indicating that other transcription factors are involved in activating transcription; and ii) *Dm myb* is not expressed in all tissues in which DREF is expressed, most notably endoreplicating larval tissues (e.g. salivary glands; Hirose et al., 1996; Yamaguchi et al., 1996), indicating that there must be at least one transcription factor acting to repress *Dm myb* transcription in these tissues.

We have also shown that in vitro and in cell culture, the efficiency of translation of the DMyb protein from a full-length transcript is reduced by the presence of seven upstream AUGs in the 5'-UTR. Based upon extensive analyses of 5' noncoding sequences, Kozak (2000) has concluded that the presence of more than four upstream AUG codons is often a warning sign of an aberrant cDNA. However, it seems unlikely that this is the case with *Dm myb* because the same 5' noncoding sequences are present in multiple independent cDNAs isolated by ourselves and others, and the start site of transcription has been supported by primer extension studies (Peters et al., 1987; and see Section 3.1). Since none of the upstream AUG codons is in an optimal context and each is followed by an in-frame terminator codon that occurs before the start of the DMyb coding domain, it is possible that the DMyb AUG may be reached by a combination of leaky scanning and translational reinitiation, or that it may depend upon the presence of an RNA element that directs internal initiation, called an IRES (internal ribosome entry site) (Jackson and Kaminski, 1995; Kozak, 1999).

The *Dm myb* transcript is not unique in having been poorly designed for efficient translation. An inspection of accessible mammalian *Myb* gene sequences revealed that the 5'-UTRs of at least human and mouse A-*Myb* and mouse c-*Myb* contain two upstream AUGs each (GenBank accession nos. AF149089, L35261, NM033597). *Dm myb* (and possibly the vertebrate *Myb* transcripts) joins a list of previously identified transcripts that encode a variety of regulatory proteins, including oncoproteins, growth factors, and transcription factors, which have lengthy 5'-UTRs containing upstream AUGs (Kozak, 2000). Transcriptional initiation and mRNA stability affect the expression levels of nearly all gene products. However, the expression of key regulatory proteins may require the introduction of an additional point at which gene expression can be regulated since excess levels of these proteins can produce deleterious effects for cell and organismal survival. The presence of unfavorable 5'-UTRs would allow for the regulation of translational initiation based upon developmental and/or environmental cues that establish cellular context.

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