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An Essential E Box in the Promoter of the Gene Encoding the mRNA Cap-Binding Protein (Eukaryotic Initiation Factor 4E) Is a Target for Activation by c-myc

ROBIN M. JONES,[1,2,3] JOHN BRANDA,[1] KELLY A. JOHNSTON,[1] MICHAEL POLYMENIS,[1] MICHELE GADD,[1,4] ANIL RUSTGI,[1]† LARRY CALLANAN,[1]‡ AND EMMETT V. SCHMIDT[1,2]*

Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts 02129,[1] and The Children's Service,[2] Department of Surgical Oncology,[4] and Department of Neurology,[3] Massachusetts General Hospital, Boston, Massachusetts 02114

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The mRNA cap-binding protein (eukaryotic initiation factor 4E [eIF4E]) binds the m^7GpppN cap on mRNA, thereby initiating translation. eIF4E is essential and rate limiting for protein synthesis. Overexpression of eIF4E transforms cells, and mutations in eIF4E arrest cells in G_1 in *cdc33* mutants. In this work, we identified the promoter region of the gene encoding eIF4E, because we previously identified eIF4E as a potential myc-regulated gene. In support of our previous data, a minimal, functional, 403-nucleotide promoter region of eIF4E was found to contain CACGTG E box repeats, and this core eIF4E promoter was myc responsive in cotransfections with c-myc. A direct role for myc in activating the eIF4E promoter was demonstrated by cotransfections with two dominant negative mutants of c-myc (MycΔTAD and MycΔBR) which equally suppressed promoter function. Furthermore, electrophoretic mobility shift assays demonstrated quantitative binding to the E box motifs that correlated with myc levels in the electrophoretic mobility shift assay extracts; supershift assays demonstrated max and USF binding to the same motif. *cis* mutations in the core or flank of the eIF4E E box simultaneously altered myc-max and USF binding and inactivated the promoter. Indeed, mutations of this E box inactivated the promoter in all cells tested, suggesting it is essential for expression of eIF4E. Furthermore, the GGCCACGTG(A/T)C(C/G) sequence is shared with other in vivo targets for c-myc, but unlike other targets, it is located in the immediate promoter region. Its critical function in the eIF4E promoter coupled with the known functional significance of eIF4E in growth regulation makes it a particularly interesting target for c-myc regulation.

All mRNA molecules are capped by a 7-methyl guanosine molecule (m^7GpppN) which is recognized by the cap-binding protein, eukaryotic initiation factor 4E (eIF4E) (33). This factor is part of an mRNA cap-binding complex which regulates attachment of the 40S ribosomal subunit to mRNAs. eIF4E is the least abundant translation initiation factor, and it is rate limiting in protein synthesis (26). Consequently, it plays an important role in the regulation of protein synthesis in the cell.

eIF4E has also been shown to play an important role in the regulation of cell growth and differentiation (69). Recessive mutations in *Saccharomyces cerevisiae* eIF4E (*cdc33*) arrest growth in the G_1 phase of the cell cycle (16). Overexpression of eIF4E transforms mammalian cells to a malignant phenotype (45). eIF4E induces mesodermal differentiation in developing *Xenopus* embryos (41). Considering that eIF4E is the least abundant translation initiation factor, is rate limiting in protein synthesis, and regulates cell growth, the regulation of eIF4E levels must be critical in growth control.

The abundance of individual translation initiation factors varies in different growth conditions (25). eIF4E is ubiquitously expressed, but its abundance increases in response to growth stimuli. Reasoning that the regulation of translation initiation factors may be particularly important at points in the cell cycle at which protein synthesis is rate limiting for growth (54), we found that levels of eIF4E mRNA indeed peaked at the restriction point in late G_1 in factor-stimulated fibroblasts (59). Increased levels of eIF4E mRNA paralleled increased c-myc levels and were markedly increased in rat embryo fibroblasts that stably overexpressed c-myc. Furthermore, activation of an estradiol-regulated chimeric myc fusion protein resulted in immediate transcriptional increases in mRNA for eIF4E.

The c-*myc* proto-oncogene has been studied as a critical regulator of cellular proliferation and as a prominent oncogene in diverse tumors (23, 40, 70). The timing of the maximum expression of myc after growth induction implicates myc as a key regulator of the growth response after mitogenic stimulation (40). In addition, sustained high levels of myc are required for the continuous growth of cells (32, 68). Consequently, myc is likely to regulate genes that mediate the mitogenic response and are important for sustained cellular proliferation.

Translocations between the three immunoglobulin loci and c-myc are always found in Burkitt's lymphomas (47). This cytogenetic evidence implicates myc as a proximal cause of cancer. Abnormalities in myc expression have also been found in a wide range of additional tumor types, and numerous experimental systems confirm the importance of myc in tumorigenesis (44). *myc* is also a potent oncogene capable of initiating transformation in diverse primary tissues in transgenic models (1, 2, 66, 71). The wide range of tissue types in which *myc* can function as an important oncogene suggests that potential myc-regulated genes may well themselves transform diverse tissue types.

* Corresponding author. Mailing address: Massachusetts General Hospital Cancer Center, Bldg. 149, 13th St., Charlestown, MA 02129.

† Present address: Gastrointestinal Unit, Division of Medicine, Massachusetts General Hospital, Boston, MA 02114.

‡ Present address: Duke University Medical Center, Durham, NC 27710.

TABLE 1. Genes regulated by c-myc

Gene	Type of regulation	Screen	Type of expression	Location of CACGTG	Reference
eIF4E (cap-binding protein)	Up	Candidate gene	Ubiquitous	Promoter	59 and this paper
Adenovirus MLP	Up and down	Candidate gene	Viral infection	Promoter	49
eIF-2α (Met-tRNA binding complex)	Up	Candidate gene	Ubiquitous	Promoter CGCATG	59
ECA39	Up	Subtraction library	Brain tumor, lymphoma, embryonal carcinoma, teratocarcinoma	Exon 1	10
Carbamoyl-phosphate synthase (*cad*)	Up	Candidate gene	Ubiquitous	Exon 1	52
α-Prothymosin	Up	Subtraction library	MYC-ER in Rat 1A	Intron 1	27 and 30
ODC	Up	Candidate gene	Ubiquitous	Intron 1	8

A number of potential targets for myc regulation have been suggested (Table 1). In particular, ornithine decarboxylase (ODC) was suggested as a candidate myc-regulated gene on the basis of known functions in growth control that are similar to functions of eIF4E (8). Like eIF4E, ODC is a delayed early gene, and it can transform cells. Mapping studies identified a dimeric CACGTG motif that fell in the first intron of the ODC gene and that was regulated by c-myc.

Major advances in our understanding of myc as a transcriptional activator have been based on structural studies (19, 20). Mutation studies identified DNA-binding and transcription activation domains as being important to the transforming functions of myc (37, 72). The DNA-binding region contains conserved motifs, including a basic region, a helix-loop-helix, and a leucine zipper (53). Additional studies identified max as the heterodimeric DNA-binding partner for myc (14). With the information gained from such structural studies being used, the binding motif CACGTG was identified as the highest-affinity target for myc binding (13, 31, 57), although less is known about the importance of the sequences that flank CACGTG.

Additional helix-loop-helix proteins bind and activate CANNTG (E box) motifs (7, 28). One abundant CACGTG-binding protein found ubiquitously in all cells is upstream factor USF that activates an essential CACGTG element in the adenovirus major late promoter (MLP) (65, 67). USF plays an important role in the basal expression of many genes (67). Both USF and c-myc bind the adenovirus MLP CACGTG site, and both interact with the basal transcription apparatus through transcription initiation factor TFII-I (60, 61). Despite their abilities to bind the adenovirus MLP site, the optimal binding sites of USF and c-myc are thought to differ slightly. Some studies suggest that they may be distinguished by altering the core CG dinucleotide within the CACGTG target. The TFE3 binding site (CACATG), for example, is an alternative binding site for myc-max (7, 12, 29, 38), but USF does not bind CACATG (57). In additional studies, binding of USF or c-myc has been distinguished by nucleotides flanking the CACGTG target sequences. A/T base pairs in the −4, −5, or −6 position flanking the core E box were preferred by USF but inhibited c-myc binding and activation (9, 29, 57).

Since levels of translation initiation factor eIF4E are important in growth control and since c-myc regulates eIF4E in several model systems, we cloned promoter sequences of eIF4E to identify important *cis*-acting elements in its regulation. We now report that this promoter contains two inverted 12-nucleotide repeats that are homologous to myc- and USF-binding motifs in the adenovirus MLP and to myc-regulated sequences in other genes, particularly ODC. The core of these sequences is CACGTG, identical to the canonical myc-binding sequence.

MATERIALS AND METHODS

Phage, plasmids, and nucleic acids. Standard manipulations of *Escherichia coli*, nucleic acids, and tissue culture cells were performed essentially as described previously (63). A human placental genomic DNA library cloned in λ FIX II (Stratagene, La Jolla, Calif.) was screened with a Klenow fragment-labeled *Taq*I-*Dra*I fragment from plasmid pTCEEC containing the human eIF4E cDNA (62) (a gift of R. E. Rhoads). Positive phage were mapped with frequent cutting enzymes to identify phage likely to contain intronic DNA segments. Phage whose frequent cutting enzyme maps suggested the possible presence of introns were mapped and subcloned into pGem 4 (Promega) with the *Taq-Dra* probe to identify appropriate phage fragments. The resulting subclones were sequenced by double-stranded dideoxy sequencing to differentiate one pseudogene-containing phage from two other phage carrying exons 2 and 3 of eIF4E. Since exon 2 of eIF4E contained sequences from nucleotides 36 to 141, we synthesized an oligonucleotide corresponding to exon 1 (ATCAGATCGATCT AAGATGGCGACTGTCGAACCG) for repeat screening of the same library. A positive phage clone was identified, and a 1,400-nucleotide *Bam*HI-*Xba*I fragment containing the exon was mapped. This fragment was subcloned into pGem 4 to make p4EBX, which was fully sequenced in both directions with appropriate subclones and synthetic oligonucleotide primers to ensure that every region was sequenced at least twice.

A plasmid for RNase protection analysis (p4EProtect) was constructed by amplifying eIF4E cDNA sequences between the *Cla*I site at position +6 and position +276 with oligonucleotide primers CCATCGATCTAAGATGGC GACTGT and CGGGATCCAGGCATTAAATTACTAG and with plasmid pTCEEC being used as a template. The PCR fragment was digested with *Cla*I and *Bam*HI; the resulting fragment was cloned into p4EBX at its *Cla*I and *Bam*HI sites to make p4EProtect. *Msc*I-cleaved p4EProtect was used as a template for SP6 RNA polymerase to generate a 370-nucleotide antisense [^{32}P]UTP-labeled RNA probe. This probe was used in RNase protections according to published procedures (66). Total RNA (30 μg) harvested from HeLa cells was used to identify the initiation site for transcription and was compared with 30 μg of yeast RNA as a negative control. The size of the protected fragment was determined with radiolabeled RNA size markers (RNA Century Marker kit; Ambion, Inc.), and parallel sequencing reactions of p4EProtect primed with CAGGCATTAAATTACTAG were used to precisely identify the start site.

The promoter region of the eIF4E gene extending from the *Xba*I site at −1042 to the *Sau*3AI site at nucleotide 1 was cloned into pBLCAT 3 (51) to make p4ECAT(1042). Nested deletions of this construct were made by a combination of subcloning at known restriction sites and exonuclease I deletions. Insertion mutations of the −403 deletion construct [p4ECAT(403)] were made by partial *Pml*I digests at the CACGTG myc box sites and religation after the insertion of *Bam*HI oligonucleotide linkers. pDiMycCAT2 was constructed by inserting an oligonucleotide linker containing

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AGCTTCGGCCACGTGACCAGTCACTAGTTGGATATCTATCCGTCACGTGGCCAGAAT
    AGCCGGTGCACTGGTCAGTGATCAACCTATAGATAGGCAGTGCACCGGTCTTAGATC
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into the *Hin*dIII and *Xba*I sites of plasmid pBLCAT 2. The expression plasmid (pMVmyc) containing myc sequences regulated by Moloney murine leukemia virus (MuLV) long terminal repeat sequences was the generous gift of James Stone (72). The expression plasmids containing dominant negative mutants of c-myc lacking the transactivation domain (amino acids 44 to 170 [pMVmycΔ TAD]) or the basic region (amino acids 353 to 367 [pMVmycΔBR]) were the generous gift of P. Farnham (52). Expression plasmid pCXUSF was the generous gift of R. Roeder (24).

For site-directed mutagenesis, a *Hin*dIII-*Eco*RI fragment containing eIF4E sequences and a small portion of the chloramphenicol acetyltransferase (CAT) gene from p4ECAT(403) was cloned into pAlter (Promega, Madison, Wis.). The Altered Sites mutagenesis kit from Promega was used to introduce mutations into the 4E sequences of pAlt4E by using single-stranded template DNA and synthetic oligonucleotides. Plasmids containing only the desired mutant site were identified by sequencing all 403 nucleotides of the eIF4E sequences to ensure

that no second-site mutations were created. The *Hin*dIII-*Xho*I fragment containing mutated promoter sequences was placed into the *Hin*dIII-*Xho*I sites of pBLCAT 3 to construct the mutant reporter plasmids indicated below (see Fig. 9).

Cells, transfections, and CAT assays. HeLa and CV1 cells were obtained from the American Type Culture Collection; rat embryo fibroblasts transfected with c-myc (REF-myc) or the neomycin resistance gene (REF-neo) were the generous gift of R. Weinberg. Cells were routinely grown in Dulbecco modified Eagle medium with 10% fetal calf serum.

Transfections were accomplished by standard calcium phosphate coprecipitation (63). For promoter mapping transfections HeLa, REF-myc, and REF-neo cells were transfected with 10 μg of eIF4E-CAT reporter constructs and 3 μg of pSVtkhGH which is not regulated by c-myc (35). We determined human growth hormone levels in supernatant media with a commercial radioimmunoassay kit (Allegro Inc.) to normalize for transfection efficiency. CAT assays were performed by thin-layer chromatography and standard methods. Cotransfections of CV1 cells to assess transactivation by c-myc were performed with various amounts of c-myc or USF vector, up to a total of 20 μg, plus empty MuLV long terminal repeat- or cytomegalovirus-containing expression vector, to a total of 20 μg of expression vector, together with 3 μg of the indicated eIF4E promoter constructs and 3 μg of pSVtkhGH. Cotransfections of HeLa cells to assess suppression of the eIF4E promoter by dominant negative myc mutants were performed with 20 μg of one of the c-myc vectors or empty MuLV long terminal repeat vector together with 3 μg of p4ECAT(403) and 3 μg of pSVtkhGH. All CAT assays were performed with duplicate points, and each experiment was repeated multiple times. For the CV1 and HeLa cotransfections, the means and standard errors for all assays are indicated (see Fig. 7).

Expression analysis of cells with Northern (RNA) and Western blots (immunoblots). Levels of expression of c-myc, USF, max, and actin in HeLa, REF-myc, and REF-neo cells were compared with immunoblots containing 50 μg of total protein harvested in TNE buffer as described previously (58, 63). Electrophoretic mobility shift assay (EMSA) extracts were similarly analyzed with 10 μg of the extracts described below. Briefly, 50 μg of cell lysate or 10 μg of EMSA extract was analyzed on a sodium dodecyl sulfate–10% polyacrylamide gel. Proteins were electroblotted onto a nylon membrane (Immobilon; Millipore) and blocked overnight in 5% dry milk. The membrane was cut according to the molecular weights of the proteins to be identified, and the identical blot was incubated with anti-myc (9E10; Santa Cruz), anti-max (polyclonal; UBI), and anti-USF (polyclonal; Michele Sawadogo) antibodies at dilutions suggested by the suppliers. A second blot with the identical quantities of lysates was probed independently with anti-actin (N-350; Boehringer) being used as a loading control. Secondary antibodies used were those included in an enhanced chemiluminescence detection kit (Amersham) and were chosen according to the species used for the primary antibodies. Exposure times for myc, USF, and max with the same film were identical. The actin blot was exposed independently.

Total cellular RNA (18) from HeLa, REF-myc, and REF-neo cells was size fractionated (10 μg per lane) on formaldehyde agarose gels, transferred to Hybond-N nylon matrices, and cross-linked by UV light. Filters were hybridized in a rapid hybridization solution (Rapidhybe; Amersham) at 65°C with eIF4E or tubulin cDNA fragments that were ^{32}P labeled by the Klenow reaction by random priming.

DNA binding assays. Nuclear extracts were prepared from 10^8 HeLa, REF-myc, and REF-neo cells during logarithmic growth for EMSAs by the Dignam method (22) and by a recent modification of the same method (52). The modified extraction buffer used (0.5% deoxycholate, 1% octyl-β-glucoside, 0.5% Nonidet P-40, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 25% glycerol) is thought to result in improved yields of c-myc and max in the lysates. Binding reactions included 3 μg of the indicated extracts. We evaluated gel shift activity first in standard EMSA binding buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5 mM $MgCl_2$, 5% glycerol [11]) with poly(dI-dC) (0.1 μg/μl) being used as a nonspecific competitor. We compared these gel shift activities with those in a HEPES binding buffer (7.1 mM HEPES [pH 7.0], 3.6 mM $MgCl_2$, 100 mM KCl, 5.7% glycerol, 0.03% Nonidet P-40 [52]) with sonicated salmon sperm DNA (0.1 μg/μl) being used as a nonspecific competitor. Complexes formed in standard binding buffer were resolved on 6% nondenaturing polyacrylamide gels containing 0.5× TBE (0.045 M Tris-borate [pH 8.0], 0.001 M EDTA); complexes formed in HEPES buffer were resolved on 4% nondenaturing polyacrylamide gels containing MOPS (morpholinepropanesulfonic acid) buffer (0.225 M MOPS [pH 7.0], 5 mM EDTA).

The radioactive dimeric myc probe contained the proximal myc site of the eIF4E promoter:

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CCAAACGGACATATCCGTCACGTGGCCAGAAGCTGGCC
GGTTTGCCTGTATAGGCAGTGCACCGGTCTTCGACCGG
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Oligonucleotides were labeled with polynucleotide kinase and [γ-^{32}P]dATP. Labeled oligonucleotide (0.1 to 0.5 ng) was used in each binding reaction. Competition experiments were performed with the indicated molar excess of unlabeled myc site oligonucleotide. Supershift experiments included 1 μl of the indicated antibodies. The anti-USF and anti-max antibodies were as described above.

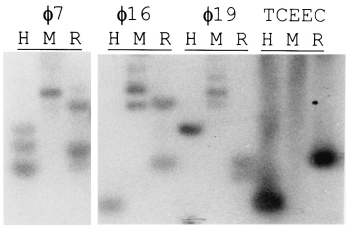

FIG. 1. Identification of sequences encoding the eIF4E promoter region. Southern blot of candidate phage DNA hybridized with the *Taq-Dra* probe containing the 5′ end of the eIF4E cDNA. The enzymes used for digestion are indicated above the lanes (H, *Hin*fI; M, *Mbo*I; R, *Rsa*I). A single band in the *Hin*fI digest of phage (ϕ) 16 is identical to the equivalent digest of the eIF4E cDNA in pTCEEC. The faster-migrating *Rsa*I band in both phages 16 and 19 migrates at the same size as that seen in the eIF4E cDNA. Phage 7 (exon 3) contains three *Hin*fI bands and two *Rsa*I bands, none of which migrates at the same size as those seen in pTCEEC, suggesting the presence of intronic DNA in phage 7.

We determined dissociation constants for USF and myc-max binding to the wild-type probe so that the three mutants could be compared (17). Bacterially synthesized USF was made with pET3dUSF (a gift of R. Roeder [39]) by following published procedures (56). In vitro gst-myc and gst-max were made by standard procedures with plasmids kindly provided by A. Rustgi and M. Billaud. Various concentrations of bacterially synthesized USF and myc-max proteins were bound to <10 pM labeled oligonucleotide (300 cpm) per reaction in the standard binding buffer (described above) without poly(dI-dC). Complexes reacting in standard binding buffer were resolved, and the free DNA (percent) was determined by densitometry. Because the concentration of DNA in these reactions is <10 pM, the protein concentration required for half-maximal binding approximates the apparent dissociation constant (K_d) for these proteins. The mutant oligonucleotides compared in these experiments were chosen on the basis of their published potential to distinguish between USF and myc-max binding (57) and included

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μN3  CCAAACGGACATATCCGTCACATGGCCAGAAGCTGGCC
     GGTTTGCCTGTATAGGCAGTGTACCGGTCTTCGACCGG

μC1  CCAAACGGACATATCGGTCACGTGGCCAGAAGCTGGCC
     GGTTTGCCTGTATAGCCAGTGCACCGGTCTTCGACCG

μ2A  CCAAACGGACATATCCGTCACGTGGAAAGAAGCTGGCC
     GGTTTGCCTGTATAGGCAGTGCACCTTTCTTCGACCGG
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where the mutant sites are in boldface type.

RESULTS

Cloning the eIF4E promoter and identification of sequence motifs. The presence of multiple eIF4E pseudogenes complicated our initial attempts to identify genomic sequences encoding the promoter region of eIF4E. Screening of a human genomic library with 5′ cDNA probes corresponding to eIF4E revealed seven phage clones that remained positive on secondary and tertiary screens. Duplicate clones of two pseudogenes were initially identified on DNA blots with frequent cutting enzymes by comparison with the eIF4E cDNA (Fig. 1); *Rsa*I cuts of these two phage identified a single band identical in size to that seen in the cDNA. After subcloning, two other phage clones proved by sequence analysis to correspond to exons 2 and 3 of eIF4E, and one additional pseudogene containing several stop codons was also identified by sequence analysis. Since exons 2 and 3 were found on separate phage clones, the size of the human eIF4E gene is obviously quite large. The phage clone encoding what appeared to be the second exon of eIF4E identified the presence of an intron at nucleotide 35 of the human 4E cDNA; we therefore rescreened the human genomic library with an oligonucleotide corresponding to the first 35 nucleotides of eIF4E and identified a phage clone containing exon 1 of the eIF4E gene. We subcloned a 1.4-kb *Bam*HI-*Xba*I fragment containing exon 1 for further studies.

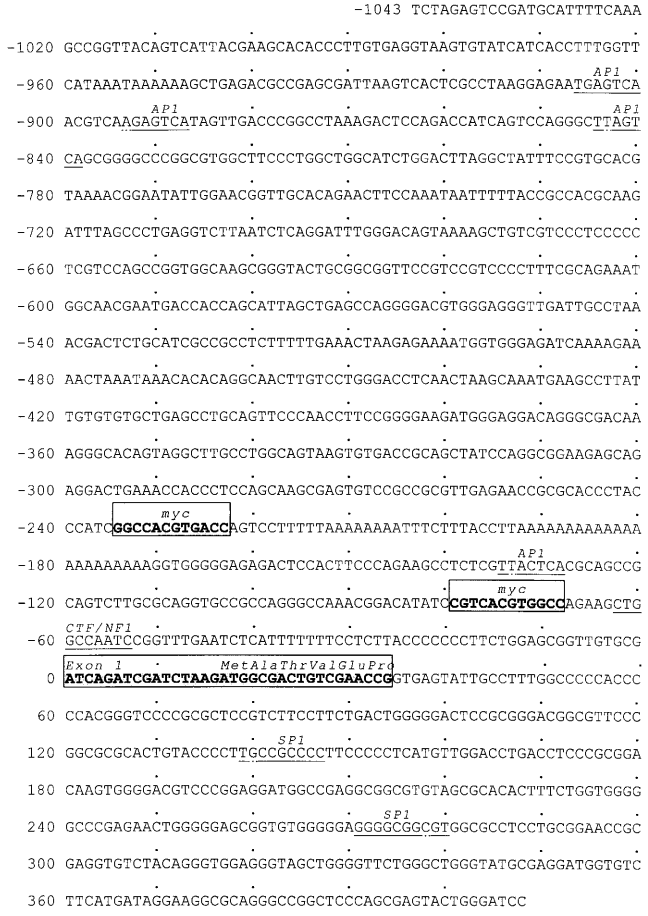

FIG. 2. Sequence of the eIF4E promoter. The sequence of the eIF4E promoter region is shown. The numbers indicate positions relative to the start site of transcription. The two potential myc protein binding sites are boxed. Potential AP1, CCAAT, and SP1 boxes identified by sequence homology are indicated. Exon 1 is boxed and designated.

We fully sequenced the subclone containing exon 1 to determine which known sequence elements might be present in the promoter of eIF4E (Fig. 2). Exon 1 nucleotide sequences in our plasmid subclone exactly corresponded to published cDNA sequences. The 3′ end of the apparent first exon contained the sequence ACCGGTGAGT, which is highly homologous to consensus splice donor sequences and contains a required GT dinucleotide.

Sequence analysis revealed several motifs in the eIF4E promoter. A canonical CCAAT box was present at position −59; TATAA sequences were not present. CACGTG sequences were found at positions −77 and −232. The sequences surrounding the upstream E box at position −232 represented a 12-nucleotide match to myc-regulated sequences found in the first intron of the ODC gene (Fig. 3) and in other myc-regulated genes. When the two elements were compared, it was apparent that the two sequences represented inverted repeats.

We identified the transcription initiation site in RNase protections with two different template plasmids. RNase protections with p4EBX demonstrated a 35-nucleotide fragment corresponding to exon 1 (data not shown). However, the signal from this short, protected fragment was weak. Consequently, we extended the probe length by subcloning an additional 269 nucleotides of cDNA sequence into the *Cla*I and *Bam*HI sites

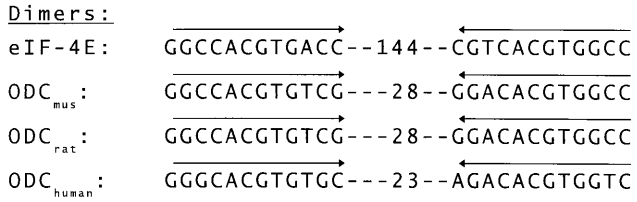

FIG. 3. Homology between eIF4E and ODC. An inverted repeat homologous to myc binding sites is present in the eIF4E promoter. The sequences of the myc dimers in the eIF4E promoter are compared with those of myc dimers present in ODC. Sequences of ODC myc-regulated sites from three species (mus = mouse) are indicated (50) and compared with the sequences present in the eIF4E promoter.

of p4EBX to make p4EProtect. With SP6 polymerase being used, a 370-nucleotide antisense probe identified a single 276-nucleotide protected fragment (Fig. 4), confirming the initiation site for transcription previously identified (34) and indicated in Fig. 2.

Promoter sequences contained in the proximal 403 nucleotides are sufficient to direct reporter gene expression. We used HeLa, REF-myc, and REF-neo cells to define a minimal promoter region necessary for directing reporter gene expression. We chose these three cell types to examine eIF4E expression in cells with various amounts of c-myc–max heterodimers but constant amounts of USF (Fig. 5). For transfections, we cloned 4E promoter sequences from the *Xba*I site to a *Sau*3AI site (positions −1042 to 3) in front of CAT sequences in pBLCAT3 (51). Sequential deletions of the promoter by standard cloning techniques (63) generated a set of deletions spanning the promoter region (Fig. 6). These reporter constructs were transfected into HeLa, REF-myc, and REF-neo cells, and promoter activity was determined by standard CAT assays. Sequences upstream of position −603 were inhibitory in this assay. The most active promoter construct contained the region between a *Pst*I site at position −403 and the initiation site. Within this maximally active promoter, deletion of the distal CACGTG box at position −230 markedly decreased promoter activity in all three cell types. Further deletion of a region containing a 23-nucleotide A-T homopolymer (positions −212 to −110) abolished CAT activity. Consequently, we identified the prox-

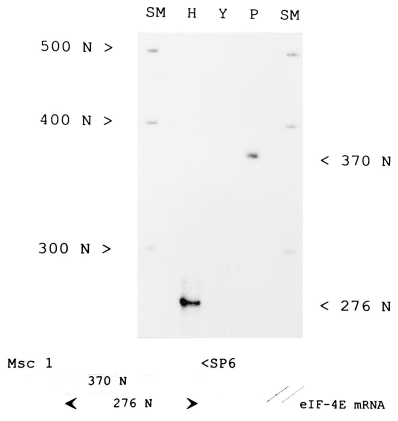

FIG. 4. Transcription initiation site. RNase protection confirms the start site for transcription initiation for eIF4E as previously reported (34). Total HeLa (H) mRNA (30 μg) was compared with yeast tRNA (Y). The size of the protected human mRNA sequence (276 nucleotides [N]) was identified from sequencing reactions run in parallel and from the indicated size markers (SM). The undigested probe (P) migrated at 370 nucleotides.

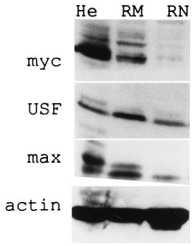

FIG. 5. Immunoblot analysis of HeLa, REF-myc, and REF-neo cells. Protein lysates (50 μg) prepared from HeLa (He), REF-myc (RM), and REF-neo (RN) cells were immunoblotted as described in Materials and Methods to compare levels of myc, USF, and max. The same lysates were probed for actin as a loading control.

imal 403-nucleotide region as a maximally active promoter, and it contained a CACGTG box that was important in activating expression of the eIF4E promoter.

The eIF4E E boxes are a target for myc regulation. We used the −403 4ECAT construct in CV1 cells in cotransfection studies to assess direct interactions with c-myc by methods previously described for artificial myc-activating sequences (43). Cotransfection with an expression vector containing c-myc sequences driven by the MuLV long terminal repeat (pMVmyc) (72) led to significant increases in the expression of CAT driven by the eIF4E promoter (Fig. 7A). Since the two CACGTG sites present in the −403 4ECAT construct were obvious myc targets, we also examined a construct containing an oligonucleotide containing these dimeric myc sites cloned upstream of the herpes simplex virus thymidine kinase minimal promoter in pBLCAT2 (pDiMycCAT2). Addition of the dimeric myc boxes conferred myc inducibility on the TAT TA-driven herpes simplex virus thymidine kinase minimal promoter (Fig. 7A). For both −403 4ECAT and DiMycCAT2, we found that increasing amounts of added c-myc vector resulted in increased CAT expression in a dose-response relationship. As was previously described (43), CV1 cells apparently express sufficient max protein to accommodate the transfected myc protein.

Deletion of c-myc's transactivation (MycΔTAD [37]) or DNA binding domains (MycΔBR [14]) results in myc mutants containing dimerization domains that still bind max but no longer function as transactivators. Since myc requires max for transcriptional activity and oncogenic function (3–5), inhibition by dominant negative myc constructs would unequivocally implicate myc and max in the activation of eIF4E. Furthermore, c-myc does not associate with USF in vitro (14), so these dominant negative mutants are unlikely to sequester USF and should strictly define a direct role for c-myc in activating eIF4E promoter expression. Finally, inhibition by MycΔBR specifically implicates max-associated factors, since MycΔTAD could potentially exclude other E box-binding proteins by binding to target E box sites. Inhibition by MycΔTAD in preference to MycΔBR would therefore identify genes regulated by non-max-associated factors (52).

We transfected p4ECAT(403) together with plasmids expressing dominant negative myc (pMVMycΔBR and pMVMyc ΔTAD) in HeLa cells to assess direct myc regulation in the highest myc-expressing target cells (Fig. 7B). Both dominant negative mutants (MycΔBR and MycΔTAD) equally inhibited 4E promoter activity. Compared with an empty expression vector (pBSltr), pMVMycΔBR inhibited p4ECAT(403) expression to 16.6% ± 4.5% of its normal activity and pMVMyc ΔTAD inhibited expression to 16.8% ± 3.1%. The functional c-myc expression plasmid (pMVmyc) increased expression by 1.6 ± 0.4-fold in these high-myc-expressing HeLa cells.

To evaluate the role of USF in activating eIF4E expression, we tested a USF expression vector in the CV1 cell assay. In contrast to cotransfection with c-myc, cotransfection with pCXUSF had no effect on the promoter (Fig. 7C).

The inverted 12-nucleotide repeats in the eIF4E promoter are binding sites for myc, USF, and max. Since c-myc levels varied among HeLa, REF-myc, and REF-neo cells, we compared eIF4E expression in these cell lines by Northern blotting (Fig. 8A). Transfection of REF cells with c-myc produced a large increase in eIF4E mRNA in a comparison of REF-myc and REF-neo cells. The additional c-myc expression in HeLa cells resulted in a smaller increase in the eIF4E level compared with that in REF-myc cells. We therefore isolated nuclear proteins from these three cell lines for use in EMSAs.

Demonstration of direct myc binding to E box motifs with in vivo nuclear extracts has proven extremely difficult (15, 50). Myc antibody experiments have only been successfully accomplished with purified preparations of c-myc and max. We compared binding with Dignam nuclear extracts and with extracts made with dialyzable nonionic detergents that have been reported to better correlate with endogenous myc levels (52). Both produced nuclear extracts containing myc levels in correspondence with endogenous levels of c-myc (Fig. 8B). We assessed binding of these extracts to the proximal E box site in the eIF4E promoter with an oligonucleotide containing the 38 nucleotides immediately surrounding the proximal myc site (Fig. 8C). A specific gel shift activity binding this E box was identified by competition with the cold oligonucleotide (Fig. 8C [large arrowhead]). Competition with other known transcription binding sites (AP1, AP2, SP1, and CTF/NF1) did not

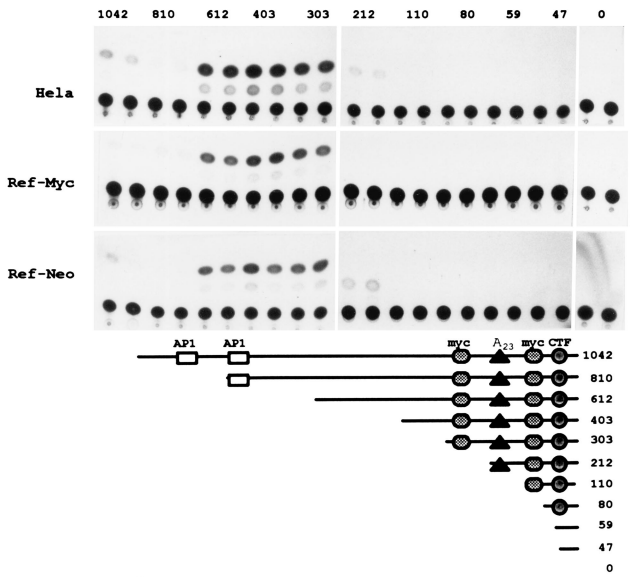

FIG. 6. A −403 4ECAT expression vector is functional in HeLa, REF-myc, and REF-neo cells. Sequential deletions of eIF4E promoter-CAT constructs reveal that the region between positions −403 and +3 is a functional promoter in HeLa, REF-myc, and REF-neo cells. The eIF4E-CAT gene constructs diagrammed at the bottom were transfected into all three cell types. Transfection efficiencies were normalized in human growth hormone assays in which pSVtkhGH was used as an internal control in each transfection. Each transfection is presented in duplicate, and the numbers correspond to the region of the eIF4E promoter included in each construct, with the lengths being given in nucleotides. This experiment was repeated three times, and a representative transfection is shown.

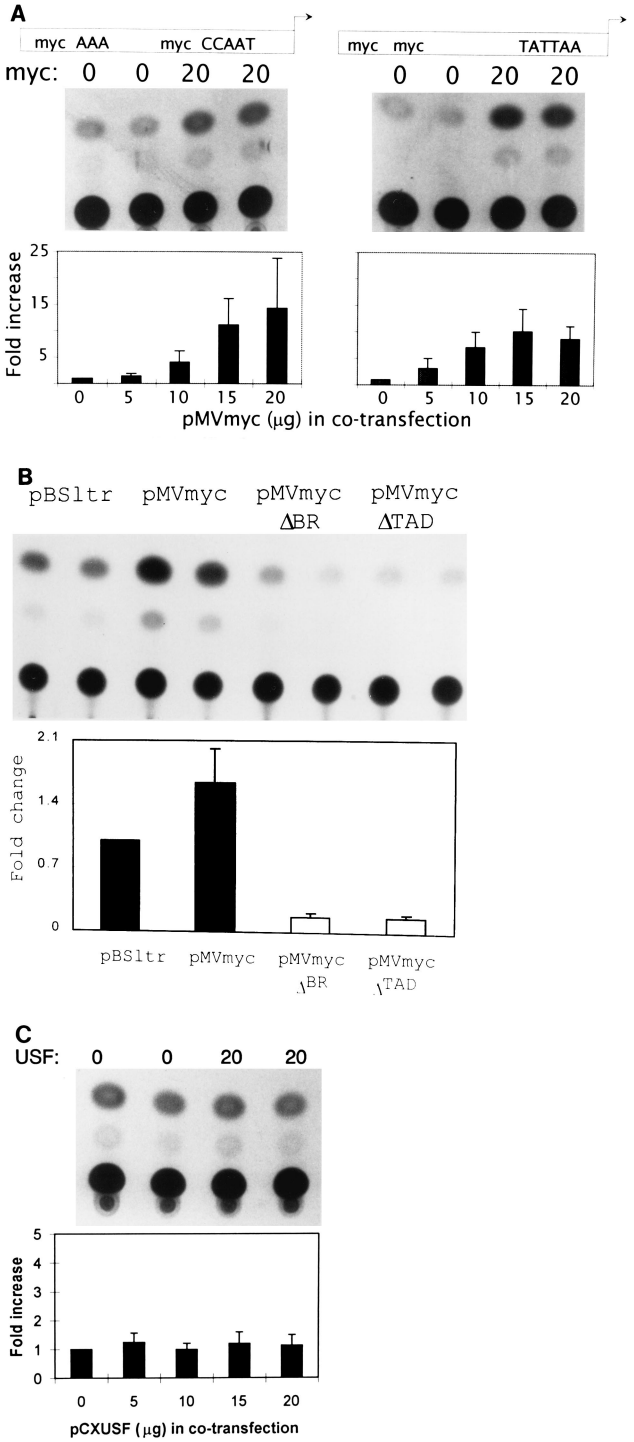

FIG. 7. The eIF4E CACGTG E boxes are transactivated by c-myc. (A) The eIF4E promoter is transactivated by c-myc. The eIF4E-CAT gene constructs diagrammed [p4ECAT(403) at left and pDimycCAT at right] were cotransfected with pMVmyc into CV1 cells. Transfection efficiencies were normalized in human growth hormone assays in which pSVtkhGH was used as an internal control in each transfection. Each transfection in the top panels is presented in duplicate, showing the effect of 20 μg of pMVmyc in the cotransfections compared with that of 20 μg of an empty MuLV expression vector. In the lower panels, we compared the dose-response effects of 0, 5, 10, 15, and 20 μg of pMVmyc (balanced with appropriate amounts of the empty MuLV vector to equalize DNA in each transfection) on p4ECAT(403) (left) or pDimycCAT (right). The means and standard errors for six transfections, each performed in duplicate, are presented. (B) Dominant negative mutants of c-myc suppress expression of the eIF4E promoter in HeLa cells. p4ECAT(403) (3 μg) was cotransfected with expression plasmids (20 μg) containing no insert (pBSltr), functional c-myc (pMVmyc), a dominant negative myc mutant lacking the DNA binding domain of c-myc (pMVmycΔBR), or a dominant negative myc mutant lacking the transactivation domain of c-myc (pMVmycΔTAD). Transfection efficiencies were normalized as described before, and a representative transfection containing duplicate assays for each transfection is displayed in the top panel. The experiment was repeated three times, and the means and standard errors for all six transfections are presented in the lower panel. (C) The eIF4E promoter is not *trans*-activated by USF. The eIF4E-CAT gene construct [p4ECAT(403)] was cotransfected with pCXUSF into CV1 cells as described above for panel A. The transfection in the top panel is presented in duplicate, showing the effect of 20 μg of pCXUSF in the cotransfection compared with that of 20 μg of an empty cytomegalovirus expression vector. In the lower panels, we compared the dose-response effects of 0, 5, 10, 15, and 20 μg of pCXUSF (balanced with appropriate amounts of the empty cytomegalovirus vector to equalize DNA in each transfection) on p4ECAT(403). The means and standard errors for three transfections, each performed in duplicate, are presented.

alter binding (data not shown). A potential role for c-myc in establishing this complex was suggested by correlation between myc levels in the EMSA extracts and the binding activity (Fig. 8C, lanes 1, 3, and 5). USF may also bind to this motif, since addition of antibodies to USF shifted the E box-binding complex to a new position (Fig. 8C, lanes 7 to 9 [small arrowhead]). This supershift was not seen with a nonspecific antibody (Fig. 8C, lanes 10 to 12 [anti-asialoglycoprotein]). Since anti-USF antibodies identify both USF and TFII-I (60), USF, TFII-I, or both may also bind to this site.

We tested these same extracts by an alternative gel shift method (Fig. 8D [52]) to demonstrate max supershifts. Once again, the main binding activity correlated with myc levels in the corresponding cells, although the difference between HeLa and REF-myc cells was less apparent (Fig. 8D, lanes 1, 3, and 5 [large arrowhead]). In the low-ionic-strength conditions used for these gels, antibodies to USF shifted the binding complex down to a new position (Fig. 8D, lanes 7, 10, and 13 [small arrowheads]). Addition of anti-max antibodies revealed a supershift (Fig. 8D, lanes 11 and 14 [large arrowheads]) that was hidden by a nonspecific band in the HeLa cells (lanes 1, 2, and 7 to 9 [asterisk]). Supershifts were not seen with the addition of anti-myc antibody (9E10) in either type of gel (data not shown). In addition, we could not further define the nature of the new complexes in the bands in lanes containing USF antibodies, because the combination of anti-USF and either normal rabbit serum or anti-asialoglycoprotein further nonspecifically shifted the supershifted band (data not shown).

The proximal E box is essential for eIF4E promoter function. We mutated both individual CACGTG sites in the −403 4ECAT construct by inserting *Bam*HI oligonucleotide linkers into blunt *Pml*I sites (Fig. 9A), since the sequence CACGTG is a recognition site for that enzyme. We also used site-directed mutagenesis to change the proximal CACGTG site to the MyoD recognition sequence CACCTG. The resulting insertion mutant of the distal −232 site inhibited promoter activity by over 80%. The linker insertion and the MyoD point mutation in the proximal −77 myc site both abolished promoter activity. Thus, we identified both CACGTG sites as critical elements, with the proximal element being essential for 4E promoter function in all three cell types.

The 12-nucleotide eIF4E E boxes, including their flanking sequences, were nearly identical to the equivalent sequences in ODC. Consequently, we compared the sequences flanking CACGTG in the eIF4E promoter with those of a variety of other genes containing CACGTG boxes thought to be activated by c-myc or USF (Table 2). Two classes of sequences were apparent. Sites that were also candidates for regulation by c-myc contained the consensus sequence GGCCACGTG

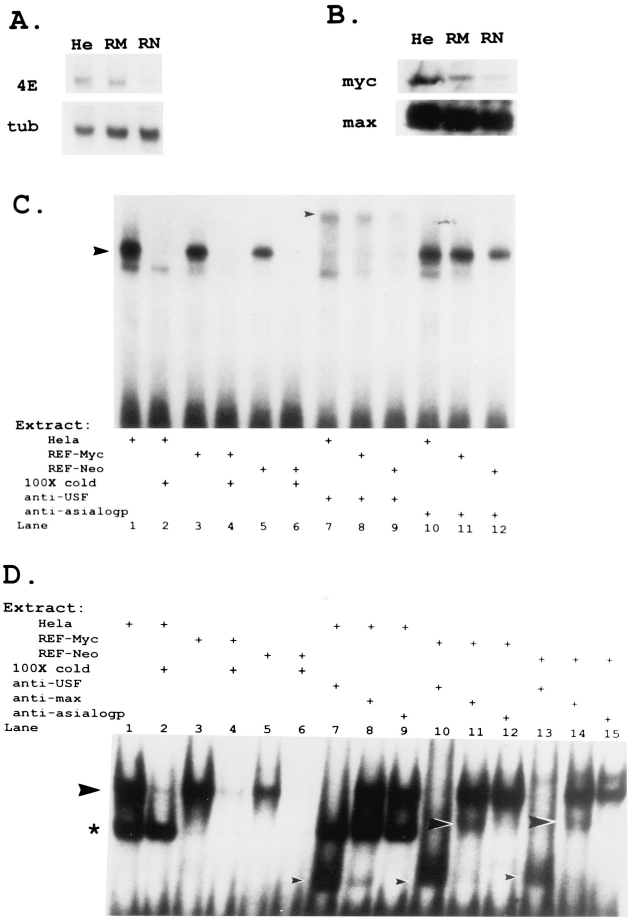

FIG. 8. EMSAs evaluate binding specificity of the eIF4E myc sites in cells expressing various levels of c-myc. (A) Northern blot analysis of eIF4E expression in HeLa (He), REF-myc (RM), and REF-neo (RN) cells demonstrates increased eIF4E expression in HeLa and REF-myc cells compared with that in REF-Neo cells. (B) Immunoblots of nuclear extracts (52) demonstrate increased myc protein in extracts from HeLa and REF-myc cells compared with that in REF-neo cells. (C) The myc site oligonucleotide

CCAAACGGACATATCCGTCACGTGGCCAGAAGCTGGCC
GGTTTGCCTGTATAGGCAGTGCACCGGTCTTCGACCGG

was radiolabeled with polynucleotide kinase and incubated with 3 μg of nuclear lysates from HeLa, REF-myc, or REF-neo cells. The protein-DNA complexes were then fractionated by electrophoresis in a nondenaturing 6% polyacrylamide gel containing TBE buffer (11) and detected by autoradiography. A cold competitor oligonucleotide contained the same sequence as the probe to demonstrate specificity. One microliter of anti-USF or anti-asialoglycoprotein (anti-asialogp) antibodies was included in the indicated lanes. The large arrowhead indicates a specific complex which correlates with myc levels in the extracts (lanes 1, 3, and 5). This complex is markedly decreased by the addition of anti-USF antibodies, and a new complex appears (small arrowhead) in lanes 7 to 9. (D) EMSAs with the identical nuclear extracts and probe were performed with a nondenaturing 6% polyacrylamide gel containing MOPS buffer (52). The specific complex again correlates with myc levels in the extracts (lanes 1, 3, and 5 [large arrowhead]). An anti-max rabbit antiserum demonstrates supershifts in the REF-myc and REF-neo extracts (lanes 11 and 14 [large arrowheads]) that were obscured by a nonspecific band (asterisk) in the HeLa cells. The USF antibody again markedly decreases specific binding in all extracts and supershifts the complex to a new position (lanes 7, 10, and 13 [small arrowheads]).

WCS. Genes that were solely candidates for activation by USF contained a T residue at −4 as predicted elsewhere (9) and contained A/T base pairs in four of the six immediately flanking residues.

We tested the significance of this prediction by making mutations in the eIF4E promoter corresponding to sequences that have distinguished between USF and myc-max binding in previous studies. Mutation of sequence CACATG is thought to inhibit USF binding more than myc-max binding; CACGTG GAA is thought to inhibit myc-max binding more than USF binding (7, 9, 12, 29, 38, 57). To compare in vitro USF and myc-max bindings to the proximal E box site, we measured the apparent K_d of the wild-type oligonucleotide used in the previous EMSAs (Fig. 9B). The apparent K_d in our measurements for USF (0.3 ± 0.1 nM) was identical to previously determined values (64). The apparent K_d for the myc-max heterodimers was lower at 8.5 ± 2 nM.

We tested the effect of *cis*-acting mutations in the eIF4E promoter that were designed to alter its flanks and core nucleotides by transfections into HeLa, REF-myc, and REF-neo cells. Three different mutations introduced into the −403 4ECAT construct abolished reporter gene activity equally in all three cell types (Fig. 9C). The apparent K_ds of these mutations were compared with that of the wild-type oligonucleotide; all three mutants altered binding of both USF and myc-max (Fig. 9C). These mutations were also totally inactive in CV1 cells and in CV1 cells cotransfected with c-myc (data not shown). Thus, our data indicated that in contrast to previous experience (7, 9, 12, 29, 38, 57), we could not identify mutations that distinguished between USF and myc-max activation of the eIF4E E box.

DISCUSSION

Regulation of translation initiation factor eIF4E. eIF4E, the mRNA cap-binding protein, is ubiquitously expressed, and *cdc33* mutations in yeast cells suggest that it is necessary for cell growth. It is essential for translation initiation of nearly all mRNAs. Consequently, its regulation should reflect aspects of its housekeeping functions. However, it is also a regulatory protein in its own right, probably by virtue of its participation in the helicase function of the eIF-4F complex. It is involved in posttranscriptional regulation of several proteins, including cyclin D1 (58), the ras pathway (46), activin (41), and mRNAs with 5′ complex structures (42). Its pleiotropic regulatory functions make it an interesting target for regulation by oncogenes that function as transcription factors (48). This characteristic led to our particular interest in clarifying the nature of the interaction between c-myc and the eIF4E promoter.

We identified a functional promoter region of the eIF4E gene that is contained within 400 nucleotides upstream of the transcription initiation site. This promoter was active in several cell types and contained two E box motifs within the immediate promoter region that were essential for its function. The location of the CACGTG E boxes in the eIF4E gene is unique, since other candidate myc-regulated genes contain E boxes in less critical sites that are not part of core promoter regions (Table 1). The adenovirus MLP contains an E box in its promoter proximal region, but MLP is biphasically downregulated by myc interactions at its initiator (INR) site (60). The E boxes in ECA39 and *cad* lie within exon 1, and neither is essential for promoter function. The E boxes in α-prothymosin and ODC lie in their first introns, and they are not essential for promoter function. The critical function of the E boxes for overall eIF4E expression therefore underlines the particular importance of c-myc expression in activating eIF4E but poses an additional dilemma, since other E box proteins, USF in particular, may also play a role in activating basal expression of the promoter.

The role of c-myc in eIF4E regulation. Levels of eIF4E increase in response to growth induction, and in our earlier study of eIF4E regulation, we identified eIF4E as a potential myc target gene (59). We now find that dimeric CACGTG

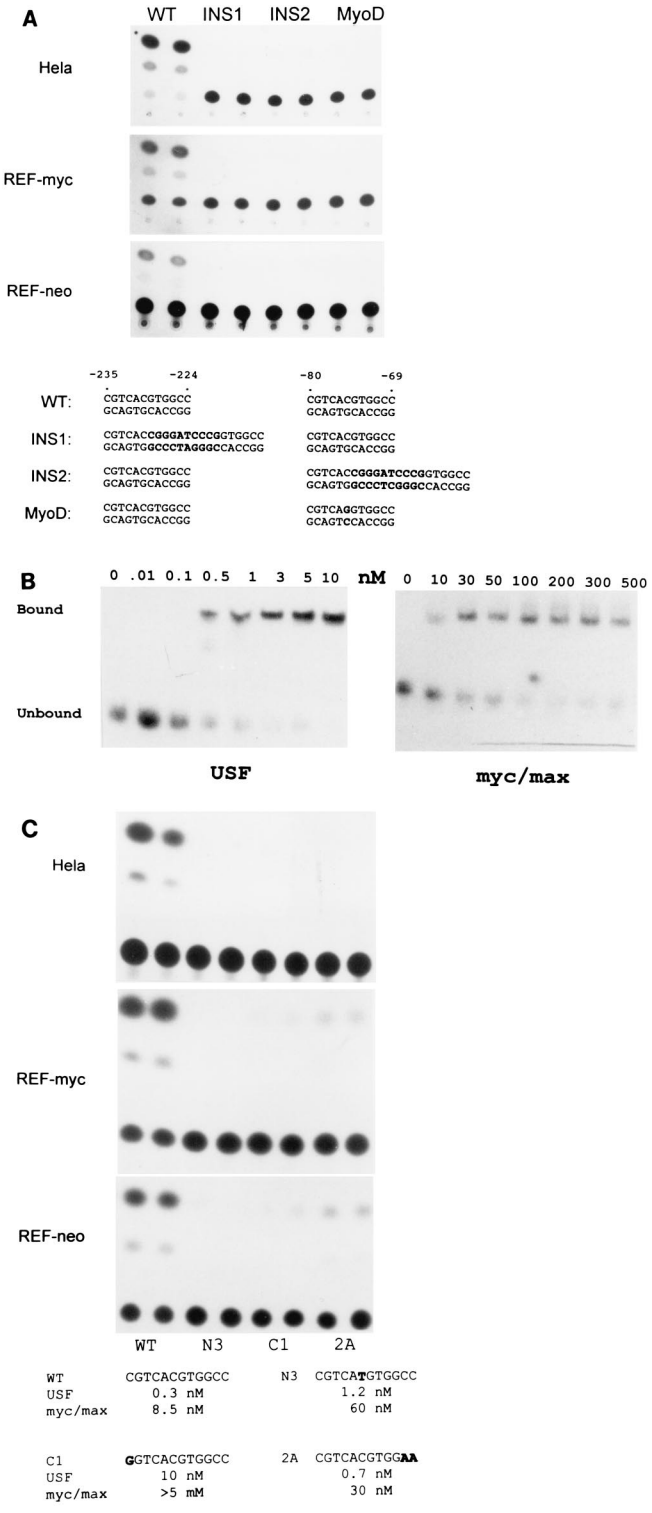

FIG. 9. A proximal myc site at position −73 is required for eIF4E expression in HeLa, REF-myc, and REF-neo cells. (A) The eIF4E-CAT gene constructs containing the mutations indicated at the bottom were transfected into HeLa, REF-myc and REF-neo cells. Transfection efficiencies were normalized in human growth hormone assays in which pSVtkhGH was used as an internal control in each transfection. Each transfection is presented in duplicate, and the experiment was repeated three times. We compared the −403 4ECAT construct (WT) with mutations of the same construct containing GGATCC insertions in the distal myc box (INS1), the proximal myc box (INS2), and a MyoD site-specific mutant containing CAGGTG (MyoD). (B) The dissociation constant of the eIF4E E box for USF was compared with that for myc-max heterodimers. The apparent K_d of the 38-mer used in our EMSAs was determined (17). The indicated concentrations (nanomolar) of bacterially synthesized USF and myc-max proteins were bound to <10 pM labeled oligonucleotide (300 cpm) per reaction. Because the concentration of DNA in these reactions is <10 pM, the protein concentration required for half-maximal binding approximates the apparent dissociation constant (K_d) for these proteins. (C) *cis*-acting mutations in the proximal E box inactivate expression of eIF4E reporter constructs in HeLa, REF-myc, and REF-neo cells. The eIF4E-CAT gene constructs containing the mutations indicated at the bottom were transfected into HeLa, REF-myc, and REF-neo cells. Transfection efficiencies were normalized in human growth hormone assays in which pSVtkhGH was used as an internal control in each transfection. Each transfection is presented in duplicate. We compared the −403 4ECAT construct (WT) with mutations of the same construct containing CGT CA**T**GTGGCC (N3 [this mutation results in CACATG in the opposite reading frame]), **G**GTCACGTGGCC (C1), or CGTCACGTGG**AA** (2A). Apparent K_d constants (determined as described above for panel B) for all three mutant oligonucleotides are indicated at the bottom.

motifs present in the eIF4E promoter were transactivated by cotransfections with c-myc, providing strong supportive evidence for our earlier hypothesis. These motifs also conferred myc inducibility on a heterologous promoter. A direct role for myc in activating the eIF4E promoter is best supported by the sixfold decrease in 4E transcription that resulted from cotransfection with dominant negative c-myc mutants. Since mutants lacking either the transactivation domain or the DNA binding domain are equally inhibitory, myc's DNA binding partner max must lie in the pathway regulating eIF4E.

Direct participation of myc in promoter activation has been difficult to prove by EMSAs, since myc antibodies are generally inactive in supershifts (15, 50). The correlation between myc levels and binding activity in our EMSAs supports the presence of c-myc in the gel shift activity binding the eIF4E E box. Supershifts demonstrating max in the same complex provide additional support. However, we could not supershift the complex with any available myc antibodies, as was previously described in other publications.

The role of USF in the regulation of the eIF4E promoter is less clear. Previous binding studies strongly suggested that both USF and myc-max bind to the identical target sequence (57). Since homozygous loss of c-myc in transgenic mice is still compatible with development to 10.5 days of gestation (21), other transcription factors are likely to provide redundant activation of CACGTG sites targeted by c-myc. USF is certainly a leading candidate for such a redundant factor. In contrast to cotransfection in the myc experiments, cotransfection with USF did not alter expression of the eIF4E promoter despite the supershift experiments revealing USF in the binding complex. We further attempted to distinguish between myc-max and USF activation of the eIF4E promoter by *cis*-acting mutations of the E box site. Several studies previously identified mutations that potentially distinguish between myc-max and USF binding (7, 9, 12, 29, 38, 57). However, quantitative gel shift studies with the eIF4E promoter sequences demonstrated equivalent decreases in myc-max or USF binding of the promoter site regardless of the mutations. The eIF4E binding site has apparently evolved to accommodate binding by either myc-max or USF.

Our data are most consistent with a simple model for activation of the eIF4E promoter in which USF supports basal expression of the eIF4E promoter and myc-max activates expression in response to growth signals. The higher affinity of USF for the E box site in the eIF4E promoter suggests that USF occupies the eIF4E E box site until a growth stimulus alters the E box occupant. This may simply result from increased levels of c-myc in response to the growth stimulus. However, additional *trans*-acting factors altered by the growth

TABLE 2. Comparison of myc and USF binding sites

Binding site and method	Sequence	No. of flanking A/T bp
myc		
PCR[a]	CACCACGTGGTG	2
In vivo[b]		
eIF-4E1	GGCCACGTGACC	1
eIF-4E2	GGCCACGTGACG	1
Adenovirus MLP	GGCCACGTGACC	1
ODC1 (mouse)	GGCCACGTGTCG	1
ODC2 (mouse)	GGCCACGTGTCC	1
ODC3 (rat)	GGCCACGTGTCG	1
ODC4 (rat)	GGCCACGTGTCC	1
ODC5 (hamster)	GGCCACGTGTCG	1
ODC6 (hamster)	GGGCACGTGTCC	1
α-Prothymosin	GGCCACGTGCTC	1
cad	AGCCACGTGGAC	2
ECA39	CGCCACGTGTCA	2
Consensus	GGCCACGTGWCS	1
	−−−−−−+++++	
	6 5 4 3 2 1 1 2 3 4 5 6	
USF		
PCR[c]	AGTCACGTGGTA	4
In vivo[d]		
Cytomegalovirus major early transcript	GGTCACGTGAAA	4
Plasminogen activator inhibitor	AATCACGTGGCT	4
TFIII A	TATCACGTGCTC	4
C4 gene	CATCACGTGGTT	4
λ2 chain	TCTCACGTGACA	4
AAV P5	GGTCACGTGAGT	3
Consensus	NRTCACGTGNNN	4
	−−−−−−+++++	
	6 5 4 3 2 1 1 2 3 4 5 6	

[a] Reference 13.
[b] See Table 1 for references for myc-regulated sites.
[c] Reference 9.
[d] See reference 67 for USF-regulated sites.

stimulus might also decrease the affinity of USF or provide additional functions that increase the affinity of myc-max heterodimers for the same site. EMSA experiments may not accurately reflect these changes, since an excess of CACGTG binding sites is included in a standard EMSA experiment, compared with the limited number of E box sites available in vivo. The difficulties encountered in demonstrating myc supershifts are explained in this model by the higher affinity of USF for myc sites in vitro. This model predicts that increases in c-myc levels after growth stimulation lead to E box occupation by myc-max in preference to USF. Alternatively, USF binding partners may be critical in altering affinities for the normal binding site for these transcription factors.

An alternative model is suggested by biochemical interactions between USF and c-myc shown in several previous studies. Although the two proteins do not form heterodimers in standard in vitro assays (56), myc directly interferes with interactions between USF and TFII-I in in vitro transcription assays (60). The model proposed in the study by Roy et al. (60) suggests that myc is a dual-function activator capable of inhibiting initiator-driven transcription while stimulating E box activation. In their model, both myc and USF bind to TFII-I to activate expression and might therefore be part of the same complex.

Biological functions of c-myc. Our study potentially provides insights into biological functions of c-myc. The functions of other candidate myc-regulated genes (ECA39 and α-prothymosin) in growth regulation remain obscure. In contrast, the mRNA cap-binding protein (eIF4E) plays an essential role in the regulation of cell growth. Some of the functions of eIF4E and ODC, another important myc target, in growth regulation are similar. ODC transforms cells if it is overexpressed in standard fibroblast assays (6). ODC is necessary for the enzymatic transfer of the 4-aminobutyl moiety of spermidine to a lysine residue in the eIF-5A translation initiation factor, producing a modified hypusine residue that is essential for eIF-5A function (55). Translation initiation factor eIF-5A is then thought to regulate formation of the initial dipeptide bond during translation initiation. eIF-5A may not always function in exactly this manner, however, since depletion of eIF-5A in yeast cells does not abolish protein synthesis (36). However, the model is attractive in that it suggests that ODC may also be involved in a translation initiation pathway.

Comparing the functions of eIF4E and ODC (mediated by eIF-5A), our studies suggest an interesting model in which myc plays a role in regulating two pathways that control initiation of protein synthesis. Since myc expression peaks at the point in G_1 when new protein synthesis is rate limiting for cell cycle progression, this model provides a potential explanation for myc's importance in growth regulation (54).

The particular sequence of the E box motif in eIF4E matches a GGCCACGTGWCS consensus that is found in other candidate myc-regulated genes but not in genes for which USF alone has been found to activate expression. This in vivo binding sequence also differs from optimal binding sequences identified by PCR for either c-myc or USF. This sequence may therefore have been selected to permit alternative binding by c-myc or USF. Its identification in the eIF4E promoter is particularly important, since this E box site is essential for both basal promoter function and activated expression, indicating an essential role for E box-binding proteins in the regulation of eIF4E.

The scarcity of myc target genes has inhibited progress in understanding its biological functions. c-*myc* is a ubiquitously expressed, transforming gene involved in growth regulation. We originally proposed that significant myc-regulated genes should therefore also regulate growth and be capable of functioning as transforming genes in a broad range of tissues. eIF4E fits both of these criteria. We have now demonstrated myc-regulated sites in the eIF4E promoter. Since the eIF4E promoter contains these sequences in the proximal region of the promoter and they are essential for promoter function, the eIF4E promoter will be extremely useful for additional future studies of the role of c-myc in growth regulation.

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Robin M. Jones and John Branda contributed equally to this work.

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