MOLECULAR AND CELLULAR BIOLOGY, June 1994, p. 3853–3862
0270-7306/94/$04.00+0

Vol. 14, No. 6

An E-Box Element Localized in the First Intron Mediates Regulation of the Prothymosin α Gene by c-*myc*

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Received 29 November 1993/Returned for modification 2 February 1994/Accepted 10 March 1994

In RAT1A fibroblasts, expression of the prothymosin α gene is under the transcriptional control of the c-*myc* proto-oncogene. We have now cloned the rat gene encoding prothymosin α and show that the cloned gene is regulated by c-*myc* in vivo. We find that regulation by c-*myc* is mediated by sequences downstream of the transcriptional start site, whereas the promoter is constitutive and not regulated by c-*myc*. We have identified an enhancer element within the first intron that is sufficient to mediate a response to Myc and Max in transient transfection assays and to activation of estrogen receptor-Myc chimeras in vivo. We find that this element contains a consensus Myc-binding site (CACGTG). Disruption of this site abolishes the response to Myc and Max in both transient and stable assays. Mutants of either Myc or Max that are deficient for heterodimerization fail to regulate the prothymosin α gene, suggesting that a heterodimer between Myc and Max activates the prothymosin α gene. Our data define the prothymosin α gene as a bona fide target gene for c-*myc*.

The c-*myc* proto-oncogene was discovered as the cellular homolog of the transforming gene of the chicken myelocytomatosis virus MC29 (16, 63). c-*myc* appears to act as a key regulator of cell proliferation; this notion is supported by the demonstration that expression of c-*myc* closely correlates with cell proliferation (23, 34, 40, 45, 66, 67) and that cells expressing c-*myc* under the control of constitutive promoter lose the ability to control proliferation in response to growth factors and often undergo apoptosis when deprived of them (29, 39).

c-*myc* encodes two nuclear phosphoproteins (1, 21, 48) that carry a number of structural and functional elements characteristic of transcription factors. The Myc protein contains both a helix-loop-helix (46) and a leucine zipper (42) motif, both of which are essential for the transforming properties of the protein (18, 59). The Myc protein binds to DNA and specifically recognizes CA(C/T)GTG sequences (termed E boxes) (11, 50). Both in vitro and in vivo, Myc heterodimerizes with a second helix-loop-helix protein, termed Max (12, 13, 49, 65). Dimerization with Max enhances the affinity of Myc to DNA and is a prerequisite for both transcriptional activation and transformation by c-*myc* (2, 3, 41). In the absence of Myc, Max forms homodimers that also recognize E-box sequences (9, 12, 37, 49) and, in contrast to Myc/Max heterodimers, repress transcription from synthetic reporter constructs that carry E boxes in their promoters (4, 33, 41, 51). In contrast to c-*myc*, Max is also expressed in quiescent cells (9, 13, 49); the relative levels of c-*myc* and Max therefore provide a model of how growth regulation of E-box-containing promoters might be achieved. Further proteins that heterodimerize with either Max (5, 69) or Myc (53, 54, 57) have been isolated and may affect gene regulation by c-*myc*.

However, little is known about the genes that might be regulated by c-*myc* and its partner proteins (7, 8, 25, 52, 62). To identify potential target genes of c-*myc*, we have previously constructed chimeric proteins that carry the hormone-binding domain of the human estrogen receptor fused to the carboxy terminus of Myc (MycER) (24). MycER chimeras associate with Max in a hormone-dependent manner and thereby allow the reversible assembly of a Myc/Max complex in vivo (48c). Using cells that express these chimeras, we have isolated by subtractive hybridization a gene, encoding prothymosin α, that is regulated by c-*myc* (25). The prothymosin α gene encodes an acidic nuclear protein (15, 17, 30, 44, 64) of unknown function that appears to be essential for cell proliferation (56). Expression of this gene has been shown to correlate with cell proliferation in a number of experimental systems (14, 17, 22, 26, 31, 58, 60, 68). Addition of estrogen to cells that express MycER chimeras, but not to control cells, leads to a rapid induction of transcription of the prothymosin α gene; this induction is resistant to inhibitors of protein synthesis, demonstrating that it is a primary response to activation of c-*myc* (25). However, the molecular basis of this induction remained obscure.

We now report the isolation of genomic clones encoding rat prothymosin α. We show that induction by c-*myc* is mediated by an enhancer sequence located in the first intron of the prothymosin α gene. This enhancer contains a consensus binding site for Myc which is both necessary and sufficient for induction by c-*myc* and repression by Max. Our data define the prothymosin α gene as a target gene for c-*myc* and provide support that c-*myc* can indeed regulate genes via CACGTG sequences in vivo.

MATERIALS AND METHODS

Manipulation of DNA. Genomic clones encoding prothymosin α were isolated from a Fisher rat genomic library (Stratagene) by using a ^{32}P-labelled rat cDNA probe (25). Purified phages were resuspended in 500 μl of SM medium (55); 10 μl of this suspension was subjected to 30 rounds of PCR using primers A (5′-GCG GAA TTC GAG GAA GAA GGT GGG GAT-3′) and B (5′-GCG AAG CTT CGT AGG AGC CTC AGC TTC-3′). All other manipulations with DNA were carried out as described previously (55). Northern (RNA) blotting (36) and gel shift experiments (12) were carried out as described previously.

Primer extension analysis. Fifty nanograms of an oligonucleotide complementary to nucleotides 126 to 146 of the rat cDNA sequence was end labelled with [γ-^{32}P]ATP and T4

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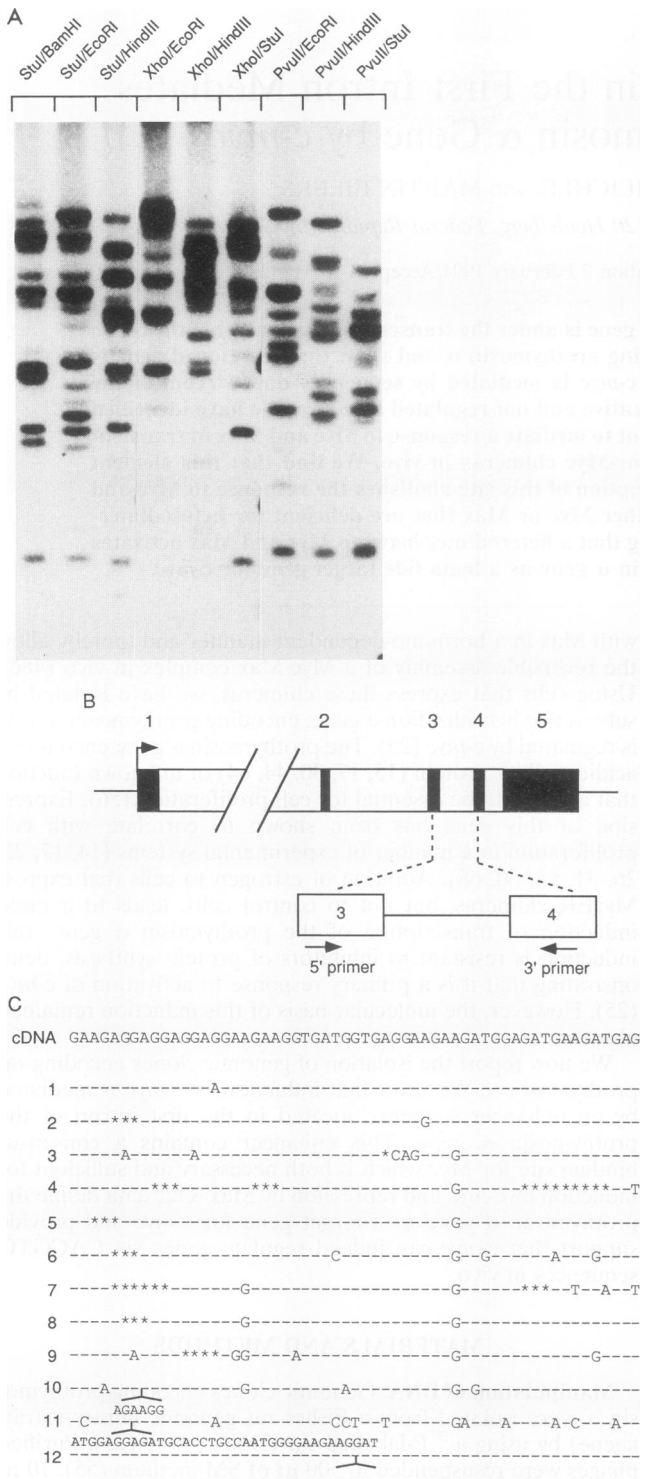


FIG. 1. Identification of the rat prothymosin α gene. (A) Southern blot of rat genomic DNA probed with rat prothymosin α cDNA. The enzymes used for digestion are indicated above the lanes. (B) PCR strategy used to identify intron-containing genes among the genomic clones. The structure of the expressed human prothymosin α gene (27) is indicated at the top; positions of the primers used for PCR amplification are indicated at the bottom. (C) Sequences obtained after subcloning the PCR products from 30 different genomic phages. Shown at the top is the rat cDNA sequence (25); numbers from 1 to 12 indicate the different genomic sequences obtained. A dash at a given position indicates sequence identity to the cDNA, and an asterisk indicates a deletion; insertions are shown with their sequences. Phage 12 is identical to the cDNA within the coding region and has an intron at the same position as the expressed human gene (27).

polynucleotide kinase to a specific activity of 2×10^4 cpm/pmol. Then 30 μg of total cellular RNA or yeast tRNA was ethanol precipitated with 50,000 cpm of primer, taken up in hybridization buffer (250 mM KCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA), and incubated for 2 min at 95°C and for 90 min at 60°C. After cooling, 10 U of avian myeloblastos virus reverse transcriptase was added in 40 μl of transcription buffer (25 mM KCl, 50 mM Tris-HCl [pH 7.5], 10 mM dithiothreitol, 3.5 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, 0.5 mM each deoxynucleoside triphosphate), and the sample was incubated for 60 min at 37°C. Nucleic acids were precipitated with sodium acetate and ethanol, and the reaction products were analyzed by electrophoresis through a denaturing polyacrylamide gel. A sequence ladder was generated by using the same primer for a sequencing reaction with Sequenase (used as instructed by the manufacturer [U.S. Biochemical]).

RNase protection assays. Templates were prepared from plasmids containing either the wild-type or mutated 1.2-kb *Hin*dIII-*Eco*RI fragment (see Fig. 2A) under the control of the T7 promoter. Plasmids were linearized with *Hin*dIII, gel purified, and diluted to a final concentration of 100 ng/μl. In vitro transcription was performed for 60 min at 37°C in a final volume of 20 μl containing 40 μCi of [α-^{32}P]UTP, 500 ng of template DNA, 500 μM each ATP, GTP, and CTP, 5 mM dithiothreitol, 40 U of RNasin (Promega), and 20 U of T7 RNA polymerase in 40 mM Tris-HCl (pH 7.5)–6 mM $MgCl_2$–2 mM spermidine–10 mM NaCl. After treatment with RQ DNase (22 U, 37°C, 30 min), the sample was extracted with phenol-chloroform, and the probe was precipitated with ammonium acetate and taken up in diethylpyrocarbonate-treated water at a concentration of 500,000 cpm/μl. For each assay, 15 to 40 μg of total cellular RNA was precipitated, washed, and dissolved in 30 μl of hybridization buffer [40 mM piperazine-*N*,*N*′-bis(ethanesulfonic acid) (PIPES)-NaOH (pH 6.4), 1 mM EDTA, 0.4 M NaCl, 80% deionized formamide]; 1 μl of the labelled RNA probe was added, and the sample was heated to 90°C for 3 min and then incubated at 46°C for 15 h. Next, 300 μl of RNase A (40 mg/ml) and 300 μl of RNase T_1 (2 mg/ml) (both in 20 mM Tris-HCl [pH 7.4]–5 mM EDTA–400 mM NaCl) were added, and samples were incubated for 1 h at 37°C. Finally, the samples were incubated with proteinase K (200 ng) and sodium dodecyl sulfate (final concentration, 0.3% [wt/vol]), extracted twice with equilibrated phenol, precipitated, and analyzed by electrophoresis in a 7.5% polyacrylamide–7 M urea gel.

Cell culture and transient transfection assays. MycER-transfected RAT1A (RAT1A-MycER) cells were grown as described previously (25). Stable transfection mixtures contained 8 μg of prothymosin α reporter plasmid and 2 μg of pSV_2hygro (kind gift of David Schatz); resistant colonies appeared in 200 mg of hygromycin per ml after 10 to 14 days and were pooled. For transient transfection assays, 3×10^5 to 5×10^5 CV-1 or HeLa cells were plated into 60-mm-diameter dishes in Dulbecco modified Eagle medium containing 10% fetal calf serum and grown overnight. Four hours before transfection, they were refed. Transfections were carried out with a standard calcium phosphate protocol (55). The total amount of DNA was kept constant in each transfection by adding equal amounts of expression plasmids. In all experi-

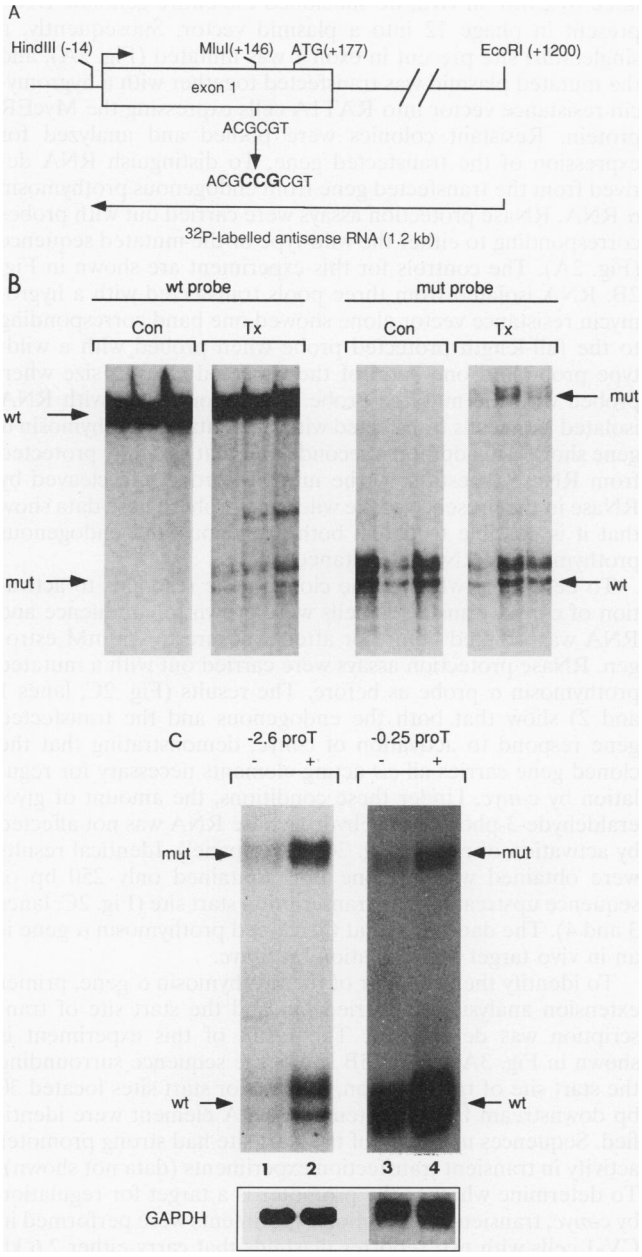


FIG. 2. The cloned prothymosin α gene is a target for regulation by c-*myc* in vivo. (A) Strategy used to distinguish RNA derived from the transfected gene from the endogenous prothymosin α RNA. The diagram shows the positions of the *Mlu*I sites used for tagging the transfected gene and the sequence of this site after mutation. In vitro transcription to generate the ^{32}P-labelled probe was performed with the *Eco*RI-*Hin*dIII fragment. (B) Pattern of protected RNAs obtained from three pools each of vector-only transfected cells (Con) or of three pools of cells transfected with the genomic tagged prothymosin α clone (Tx). RNase protection assays were carried out with either the wild-type (wt) or mutated (mut) probe, as indicated. Positions of the upper bands correspond to the expected sizes of 224 and 220 bp; those of the lower bands correspond to the expected size of 150 bp. The other fragment expected upon cleavage (70 bp) was also detectable on the original autoradiogram (not shown). (C) RNase protection assays from RAT1A-MycER cells stably transfected with tagged genomic prothymosin α clones carrying either 2.6 kb (lanes 1 and 2) or 250 bp (lanes 3 and 4) of sequence upstream of the start site of transcription. RNA was isolated from confluent cells before (−) or after (+) exposure to 200 nM estrogen for 24 h and probed with an antisense probe corresponding to the mutated (transfected) allele. The clone containing 250 bp of promoter sequence was expressed at lower levels relative to the endogenous gene; therefore, the endogenous RNA is overexposed on the autoradiogram. As a control, equal aliquots of the RNA were subjected to Northern blotting and probed for the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

ments, RSV-βgal (kind gift of G. Schütz) was used to control for transfection efficiencies; expression of this promoter was not significantly affected by either CMV-MYC or CMV-MAX expression vectors. After 24 to 48 h, cells were harvested and subjected to three freeze-thaw cycles. Luciferase and galactosidase activities were determined as described previously (38). Each assay was performed three times independently, and each experiment was repeated at least twice. Western blots (immunoblots) of transiently transfected cells were prepared as described elsewhere (36), using monoclonal antibody 3C7 (28).

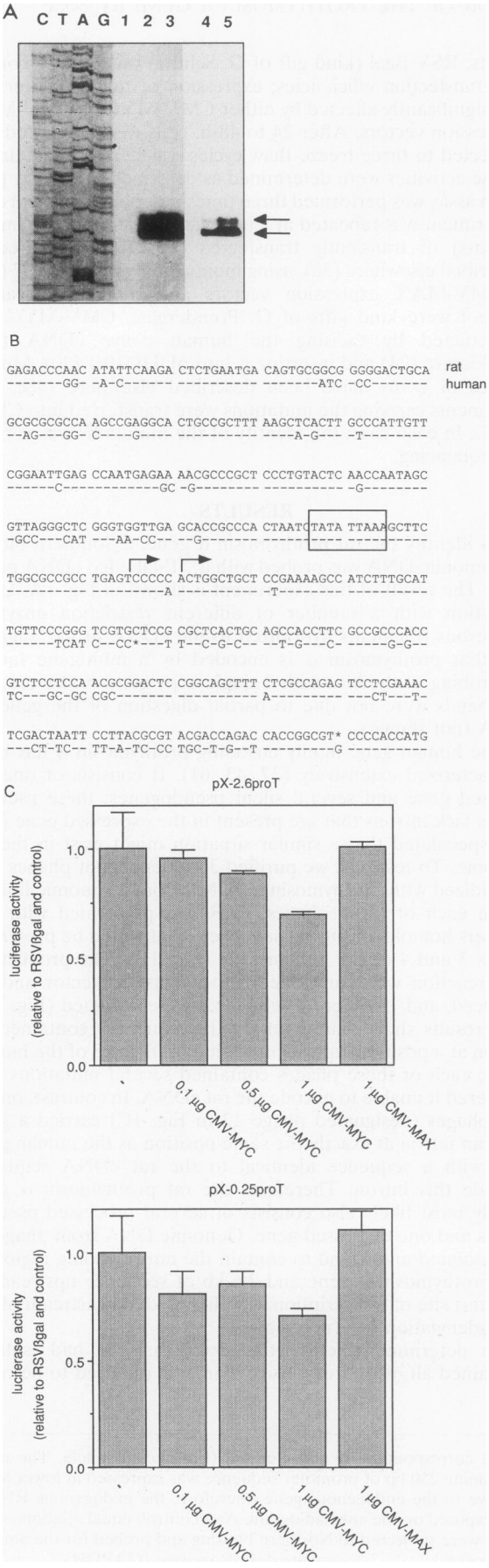
CMV-MAX expression vectors and mutant derivatives thereof were kind gifts of G. Prendergast. CMV-MYC was constructed by excising the human c-*myc* cDNA from pSP65cmyc (24) and inserting it into pUHD10-1 (20). Mutant alleles of c-*myc* have been described elsewhere (48c, 59). Fragments carrying the mutations were transferred into CMV-MYC. In each case, the identity of the mutations was verified by sequencing.

RESULTS

To identify the rat prothymosin α gene, a Southern blot of rat genomic DNA was probed with a ^{32}P-labelled cDNA probe (25). The result of this experiment is shown in Fig. 1A. Upon digestion with a number of different restriction enzymes, numerous fragments hybridized to the cDNA probe, suggesting that prothymosin α is encoded by a multigene family. Reprobing of the blot with a single copy gene confirmed that the bands were not due to partial digestion of the genomic DNA (not shown).

The human gene family encoding prothymosin α has been characterized extensively (27, 43, 61). It consists of one expressed gene and several silent pseudogenes; these pseudogenes lack introns that are present in the expressed gene (27). We speculated that a similar situation might exist in the rat genome. To test this, we purified 30 independent phages that hybridized with prothymosin α cDNA from a genomic library. From each of these phages, PCR was performed with two primers homologous to rat sequences that would be present in exons 3 and 4 of the human gene (Fig. 1B). The products of this reaction were subcloned into a plasmid vector and sequenced, and 12 different sequences were obtained (Fig. 1C). The results show that 11 of the 12 sequences contained no intron at a position equivalent to that of intron 3 of the human gene; each of these phages contained several mutations that rendered it unable to encode the rat cDNA. In contrast, one of the phages (designated phage 12 in Fig. 1C) carried a gene with an intron at exactly the same position as the human gene and with a sequence identical to the rat cDNA sequence outside this intron. Therefore, the rat prothymosin α gene family most likely also consists of several processed pseudogenes and one expressed gene. Genomic DNA from phage 12 was isolated and found to contain the entire coding region of the prothymosin α gene and 2.6 kb of sequence upstream of the start site of transcription as well as 2 kb downstream of the polyadenylation site (not shown).

To determine whether the clone that we had isolated contained all of the responsive elements required to be regu-



lated by c-*myc* in vivo, we subcloned the entire genomic DNA present in phage 12 into a plasmid vector. Subsequently, a single *Mlu*I site present in exon 1 was mutated (Fig. 2A), and the mutated plasmid was transfected together with a hygromycin resistance vector into RAT1A cells expressing the MycER protein. Resistant colonies were pooled and analyzed for expression of the transfected gene. To distinguish RNA derived from the transfected gene from endogenous prothymosin α RNA, RNase protection assays were carried out with probes corresponding to either the wild-type or the mutated sequence (Fig. 2A). The controls for this experiment are shown in Fig. 2B. RNA isolated from three pools transfected with a hygromycin resistance vector alone showed one band corresponding to the full-length protected probe when probed with a wild-type probe and one band of the expected cleaved size when probed with the mutated probe. Protection assays with RNA isolated from cells transfected with the mutated prothymosin α gene showed in addition a second band that was fully protected from RNase digestion by the mutated probe and cleaved by RNase in the presence of the wild-type probe. These data show that it is possible to detect both exogenous and endogenous prothymosin α RNAs simultaneously.

To determine whether the cloned gene responds to activation of c-*myc*, transfected cells were grown to confluence and RNA was isolated before or after exposure to 250 nM estrogen. RNase protection assays were carried out with a mutated prothymosin α probe as before. The results (Fig. 2C, lanes 1 and 2) show that both the endogenous and the transfected gene respond to activation of c-*myc*, demonstrating that the cloned gene carries all *cis*-acting elements necessary for regulation by c-*myc*. Under these conditions, the amount of glyceraldehyde-3-phosphate dehydrogenase RNA was not affected by activation of c-*myc* (Fig. 2C, lower panel). Identical results were obtained with a clone that contained only 250 bp of sequence upstream of the transcription start site (Fig. 2C, lanes 3 and 4). The data show that the cloned prothymosin α gene is an in vivo target for regulation by c-*myc*.

To identify the promoter of the prothymosin α gene, primer extension analysis was carried out and the start site of transcription was determined. The result of this experiment is shown in Fig. 3A. Figure 3B shows the sequence surrounding the start site of transcription. Two major start sites located 30 bp downstream from a potential TATA element were identified. Sequences upstream of the start site had strong promoter activity in transient transfection experiments (data not shown). To determine whether the promoter is a target for regulation by c-*myc*, transient transfection experiments were performed in CV-1 cells with two reporter plasmids that carry either 2.6 kb or 250 bp of sequence upstream of the transcriptional start site in front of a luciferase reporter gene. In these experiments, we

FIG. 3. The prothymosin α promoter is not a target for regulation by c-*myc*. (A) Primer extension analysis demonstrating the two major start sites of transcription observed in RAT1A cells. Lane 1 contains a control with yeast tRNA; lanes 2 and 3 contain 5 and 15 μg each of total cellular RNA; lanes 4 and 5 are shorter exposures of lanes 2 and 3, respectively. (B) Sequence of the rat prothymosin α gene around the start site of transcription in comparison with the human DNA sequence (27). The fragment shown corresponds to the upstream sequence present in the clone used for the RNase protection assays shown in Fig. 2C (lanes 3 and 4). Arrows indicate the start sites of transcription. (C) Transient transfection assays in CV-1 cells with reporter plasmids carrying either a 2.6-kb (pX-2.6proT) or 250-bp (pX-0.25proT) promoter fragment in front of a luciferase reporter gene. Shown is the response to both CMV-MYC and CMV-MAX.

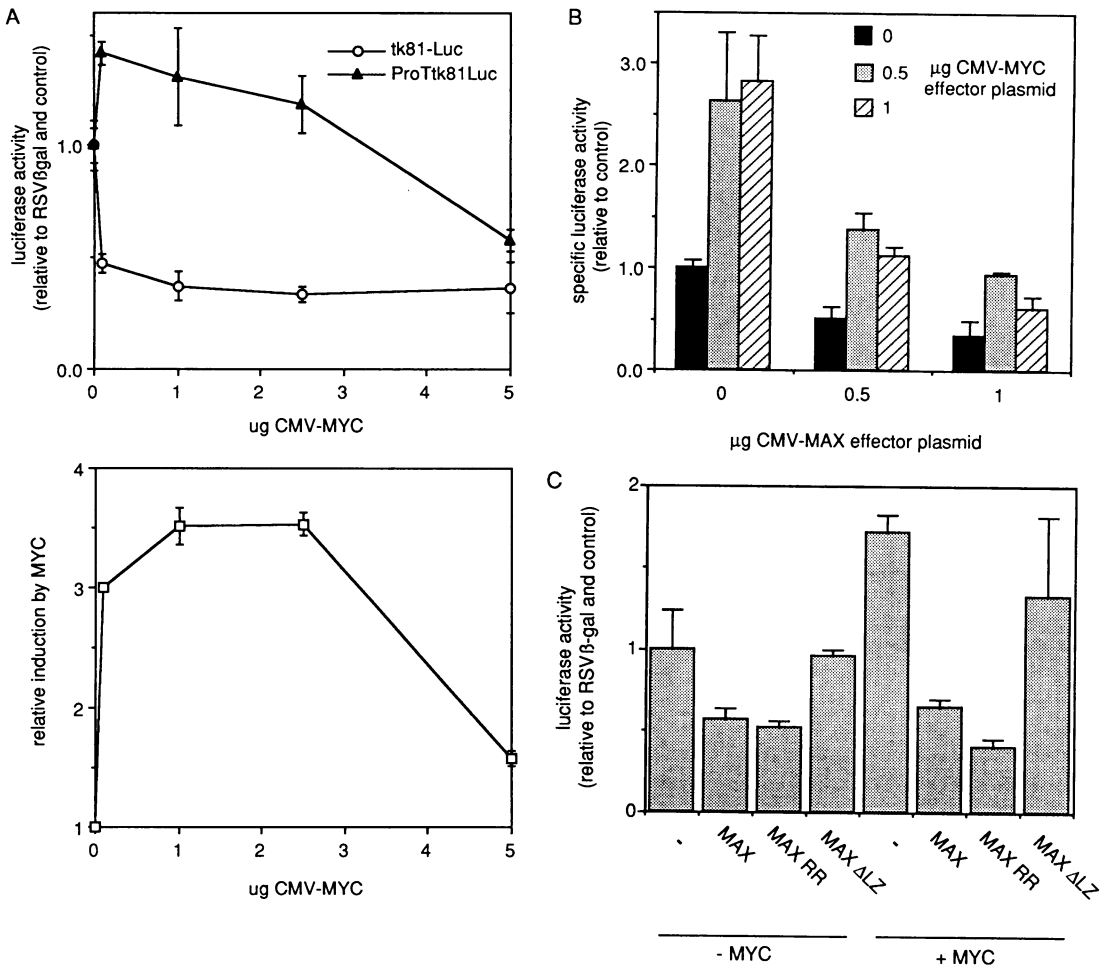


FIG. 4. Sequences downstream of the start site of transcription mediate regulation by c-*myc* and Max. Shown are transient transfection assays with either tk81luc or ProTtk81luc as the reporter plasmid in CV-1 cells. (A) Response of tk81luc and ProTtk81luc to increasing amounts of CMV-MYC (upper panel). The lower panel shows the induction by expression of c-*myc* of ProTtk81luc relative to tkluc. (B) Titration of both CMV-MYC and CMV-MAX on ProTtk81luc. (C) Effects of different mutants of Max on expression of ProTtk81luc in the absence (−MYC) or presence (+MYC) of CMV-MYC. In each experiment, 0.5 μg of effector plasmid was used. MaxRR carries two point mutations in the basic domain that abolish its ability to bind to DNA; MaxΔLZ carries a deletion of the leucine zipper (49).

consistently observed a slight inhibition by cotransfection of c-*myc*; no significant response to expression of Max was seen (Fig. 3C). A control experiment using a synthetic reporter plasmid that carried multiple E boxes in front of a simian virus 40 minimal promoter showed that this construct was activated fivefold by expression of c-*myc* relative to a similar reporter lacking E-box sequences (not shown). When stably reintroduced into RAT1A-MycER cells, the prothymosin α promoter had strong constitutive activity that was not down regulated in confluent cells and did not respond to addition of estrogen (see Fig. 6A; also data not shown). We conclude that the promoter of the prothymosin α is not a target for regulation by c-*myc*.

Therefore, we wished to determine whether sequences downstream of the start site of transcription contain a *cis*-responsive element that is regulated by c-*myc*. Sequences downstream from the transcriptional start site were cloned in front of a herpes simplex virus type 1 thymidine kinase minimal promoter in a luciferase reporter plasmid (tk81luc, generating ProTtk81luc) (47). This plasmid was transiently transfected into CV-1 cells together with increasing amounts of a CMV-MYC expression vector. As a control, tk81luc was transfected into the same cells. The results are shown in Fig. 4A.

Even at low concentrations, expression of c-*myc* leads to a two- to threefold repression of expression from tk81luc relative to control transfections. Similar results were obtained with a simian virus 40 minimal promoter, suggesting that c-*myc* can repress core promoter elements in these transfection assays (not shown; see also Fig. 3C) (1). In contrast, ProTtk81luc was stimulated by low concentrations of c-*myc*; relative to tk81luc, expression of c-*myc* mediated a three- to fourfold activation of the prothymosin α promoter, similar to the fivefold regulation by MycER observed in vivo (25). We conclude that sequences downstream of the transcription start site mediate activation by c-*myc*.

To test whether expression of prothymosin α is affected by Max, the experiment was repeated in the presence of increasing amounts of a CMV-MAX expression plasmid. Expression of Max did not affect the activity of the tk81luc reporter plasmid (Fig. 5A). The results obtained with ProTtk81luc (Fig. 4B) showed that expression of Max reduced the basal activity of this construct and abolished transactivation by c-*myc* in a dose-dependent manner. Repression by Max required an intact leucine zipper but not an intact DNA-binding domain (Fig. 4C), suggesting that Max could repress expression of

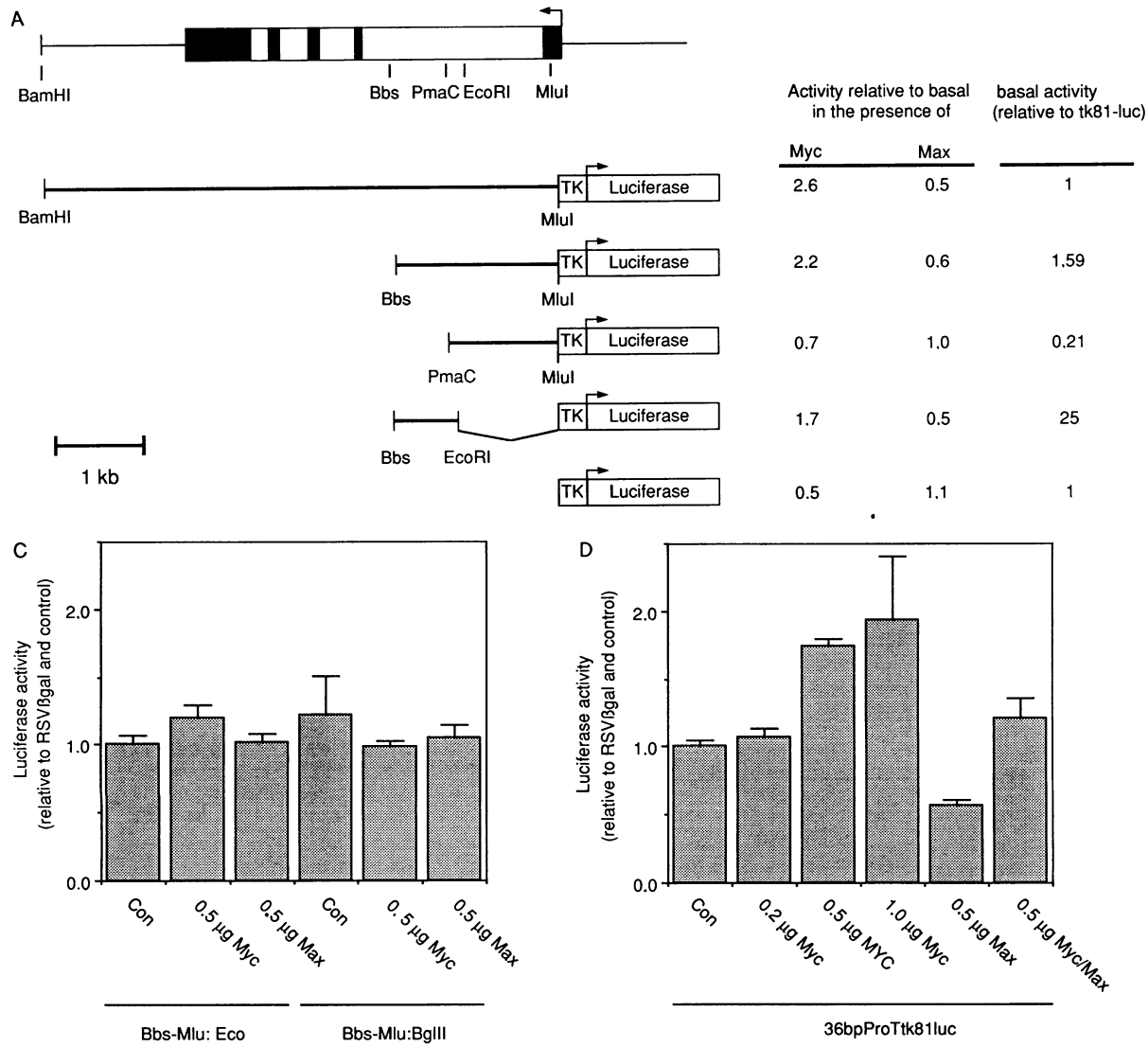


FIG. 5. The *cis*-responsive element for regulation by c-*myc* is localized within the first intron. (A) Transient transfection assays with a series of deletion mutants of ProTtk81luc. Indicated at the right is the degree of transactivation by CMV-MYC and repression by CMV-MAX relative to the basal activity of each reporter and the basal activity of each reporter relative to tk81luc. (B) Sequence of the 1,109-bp *Bbs*I-*Eco*RI fragment shown in the fourth line of panel A. The consensus Myc-binding site is boxed; the 36-bp oligonucleotide used as a reporter in panels C and D is underlined. (C and D) Transient transfection assays with either CMV-MYC or CMV-MAX. (C) The CACGTG sequence was disrupted within the *Bbs*I-*Mlu*I reporter plasmid by insertion of either an *Eco*RI or *Bgl*II linker. (D) A 36-bp oligonucleotide spanning the CACGTG site was synthesized and inserted into tk81luc. Shown is the response to increasing amounts of CMV-MYC and to CMV-MAX.

ProTtk81luc either by forming homodimers or by sequestering Myc upon formation of nonfunctional heterodimers. Similarly, mutant alleles of Myc that are deficient for dimerization with Max failed to activate the prothymosin α gene (see Fig. 7), suggesting that prothymosin α is indeed activated by a heteromeric Myc/Max complex (see Discussion).

A series of deletion constructs was used to localize the *cis*-responsive elements within the prothymosin α gene. Figure 5A indicates which constructs could be activated by c-*myc* and repressed by Max and shows the basal activities of these constructs relative to tk81luc. The results show that the c-*myc*-responsive element is localized within a 1,109-bp *Bbs*I-*Eco*RI fragment contained within the first intron. The data also demonstrate that intron 1 contains at least two distinct elements that affect basal promoter activity: first, a silencer element is located within the 5′ half of the intron, and second, an enhancer element overlaps the c-*myc*-responsive element. The sequence of the *Bbs*I-*Eco*RI fragment was determined (Fig. 5B) and found to contain a single consensus Myc-binding site (CACGTG; boxed in Fig. 5B). Two experiments were performed to demonstrate that this element is both necessary and sufficient to mediate the response to c-*myc*. First, the E box was disrupted in a reporter plasmid that carried the *Bbs*I-*Mlu*I fragment (Fig. 5A) by insertion of two different oligonucleotides within the central CACGTG. Second, a 36-bp oligonucleotide spanning this E box was synthesized (underlined in Fig. 5B) and cloned in front of tk81luc. Transient transfection assays were performed as before, and the results are shown in Fig. 5C and D. The data demonstrate that disruption of the CACGTG sequence abolishes the response of the *Bbs*I-*Mlu*I

B

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1    AGTCTTCAGA ACAGAGGGTA CCCATCAGCC AAAGTGCTGA AAACTAAACA
51   AAGCTGACTT ACAGAACTAA TGTGTCACTG GCTACTTAGC CACCCTGGAG
101  TGTTAGAGGA TGGGCCATAG ATCTCTCTAC TCAAGGCCTG TATCCAAATC
151  CTGACTCCCA TCTTCTGGAT ACATCGAAAA CAAGGCAGTC AGGTGTTGAC
201  TCAAGGCTCA CATATGGCAC CACAGCCACA AACCCGCCAG CCAGGCAGAC
251  AAGCTCGGGA ACTGTCTCCC TCACCTGCAC GCCCTTTGCC GCCGGTTTCC
301  AGCTAGAGGG AGCTCCACCC CTCAGAAACC CTTCTTGGAA GGCGGTGGCT
351  GTGTGCATAA ATAAATAAGG TATTGAAGGG AGGAGCCAGT CGATGGTCAG
401  TAGCTCAGGG ATGGATGGGG AGAATCTGGA TGGAGATCAG GAAGCGCTTG
451  AGCATGCAGA CACCCCCCAC TTCCAAATCA CCAGTCATGC TTGGTACTAC
501  AGGCCTTCCT AGCCTCCATA ACCCAAAGCA AGTAGCAAGC TAGCACTTTG
551  GAAACTGGCG GCTTCGGGGC GGGACCGTGT GTAACTTTTC CACTCCATGG
601  CTCAACGGTG GGACCACCAG GAAGGCCAGG CACATAGTCG AGCTAGTCCT
651  GCGGGGCCCA CCCCCTGGGT TACCTGCTGG GGTTGGTGGG TTCGCGCCCA
701  CCGAAGGCCT GCGTCGGACC CCATTGCACC CGTTCCGCCC CTGGTGCCGA
751  AGCCCGGGGG AGTGGGTGGG GGCGGGGTGG CTCAGGCGCA CCGCTCACCC
801  AGAGAACTAC GCGGCGAGCC TCGGCACGCA CCGCGCGCAA CGAGCACGTG
851  GCCTGGGGCG CCAAGTGCGC CCTGTGCCCT GGAGACCCAA GGTACGCTTT
901  CATCTAGGTG AGCGAGTTTG CCGCTGGAG GAAAGCGCGC TACGTGCTGCC
951  CCCTTGGAT CGCGACTGAG GGTCAACACG CGGCTTCGGG CTGCAATTTTG
1001 GCGCGCAAC GTCCGGCGTT AAGTCACCGG GGTATTGGAG CTTCTGAGCTC
1051 GGAGCCTCG AGACCGTCGG GGGCGCACTC CCCAGAAGGG TCGAACTCGCG
1101 GGAATTC
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FIG. 5—*Continued.*

reporter plasmid to c-*myc* and Max (Fig. 5C). In contrast, a short oligonucleotide spanning this sequence mediates a response to transfection of either effector plasmid (Fig. 5D). Similar results were obtained when this element was cloned in front of a 250-bp fragment of the prothymosin α promoter (not shown). Taken together, these data define an E box localized within the first intron as the c-*myc*-responsive element of the prothymosin α gene. The basal activity of the 36-bp reporter construct was comparable to that of the entire *Bbs*I-*Eco*RI fragment, showing that the enhancer element localized in the first intron overlaps the c-*myc*-responsive element. Gel shift experiments with either Max or the DNA-binding domain of Myc (ΔMyc) synthesized in a reticulocyte lysate demonstrated that both proteins recognize this element with high affinity (data not shown).

To show that the element that we have identified is regulated by Myc in vivo, we stably integrated the reporter plasmids shown in Fig. 5A into RAT1A-MycER cells and tested whether they were activated in response to addition of estrogen. RAT1A-MycER cells were cotransfected with the indicated plasmids and a hygromycin resistance plasmid. Resistant colonies were selected, pooled, and grown to confluence (25). Estrogen or, in control experiments, ethanol was added, and cells were harvested 8 h later. The results (Fig. 6A) show that reporter constructs carrying an intact but not a disrupted E-box element are activated by MycER in vivo. The promoter of the prothymosin α gene does not respond to activation of MycER (two leftmost bars). These results confirm those obtained in transient transfection assays; they show that the E-box element in the first intron is both necessary and sufficient to mediate a

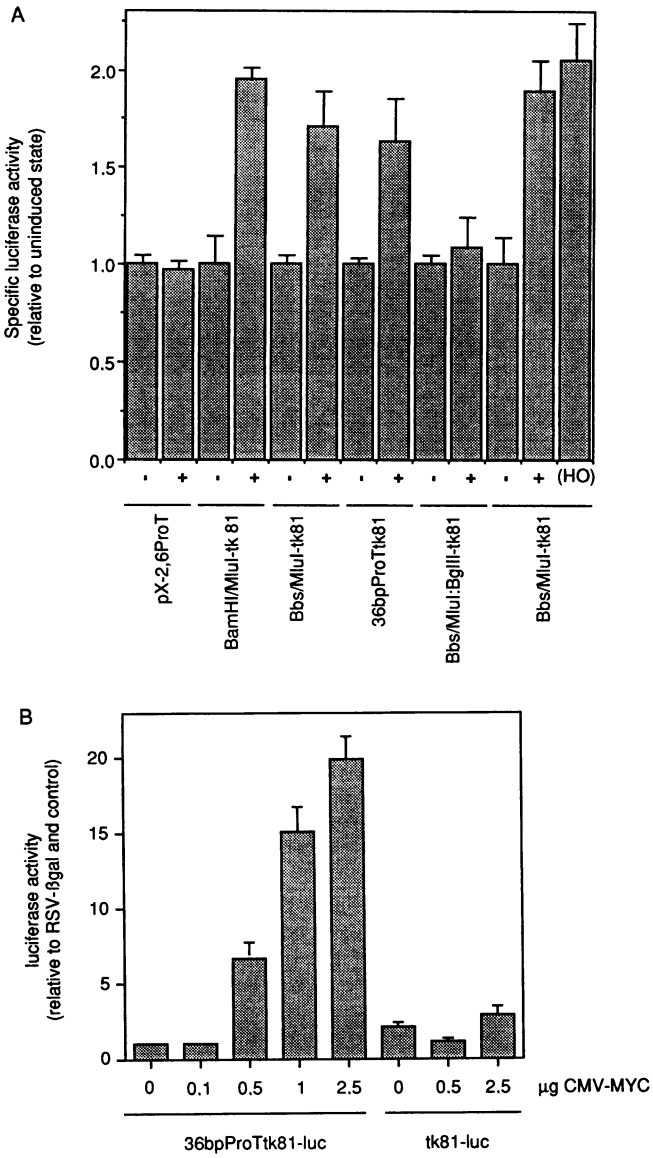

FIG. 6. Activation by c-*myc* occurs in vivo and is cell type specific. (A) Activation by MycER in vivo. The indicated reporter plasmids (see Fig. 5A) were stably integrated into RAT1A-MycER cells (see Materials and Methods). Shown is the specific luciferase activity in response to addition of 200 nM estrogen (+) relative to untreated cells (−); cells designated HO (rightmost lane) were treated with 200 nM hydroxy tamoxifen instead of estrogen. (B) Transient transfection assays in HeLa cells. Shown are the results of transient transfection assays with the indicated reporter plasmids.

response to Myc in vivo. Activation in MycER cells was observed both in response to estrogen (leftmost lanes) and in response to hydroxytamoxifen (rightmost lane), similarly to what is observed for the endogenous gene (25).

We were concerned about the low level of activation by Myc observed in CV-1 cells; to determine whether activation of this element by Myc might be cell type specific, we transfected reporter constructs carrying the intron E-box element into several different cell lines. We observed that the degree of stimulation of this element by Myc varied strongly among different lines; in particular, a 20-fold stimulation was observed

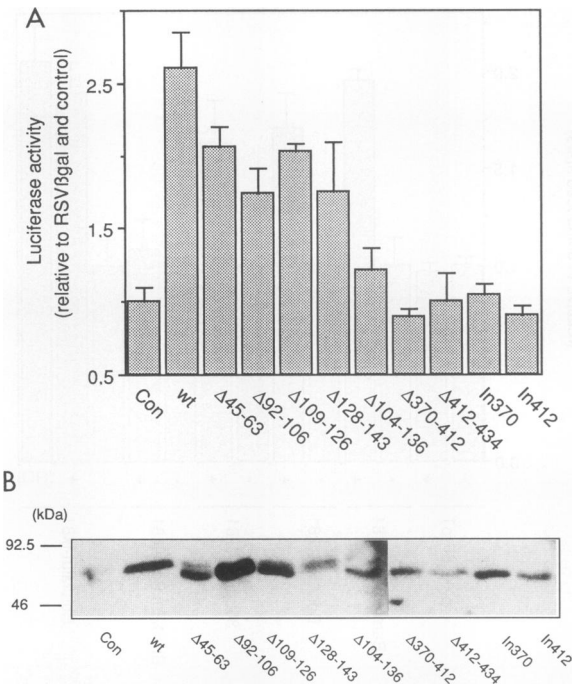

FIG. 7. Domains of Myc required for transactivation of prothymosin α. (A) Results of transient transfection assays with the 36-bp E-box reporter construct in CV-1 cells. The amino acids mutated in each construct are indicated below each bar. (B) Western blot using monoclonal antibody 3C7 (28) documenting expression of the different alleles of Myc after transient transfection into CV-1 cells.

C A C G T G G C C T G G G G C G	prothymosin-α
C A C G T G C C C G G G G C C C	human ODC MycBox1
C A C G T G T C G C G A G G C C C G	rodent ODC MycBox1
C A C G T G C C C C G G G G C C	hamster ODC MycBox2
C A C G T G G C C G G A G G C T	rat ODC MycBox2
C A C G T G G C/C C/G N G G/A G/C C	consensus

FIG. 8. Sequence comparison of several Myc response elements. Shown are the sequences surrounding the core CACGTG sequence from the Myc response elements of the prothymosin α gene and of human, rat, and hamster ornithine decarboxylase genes (7) together with a potential consensus sequence.

in HeLa cells (Fig. 6B). Our data suggest that the element that we have identified mediates a cell-type-specific response to Myc; this is reminiscent of the behavior of the endogenous gene, which is regulated by c-*myc* in RAT1A, but not BALB/c 3T3 cells (23a).

Finally, we wished to determine which domains of Myc are involved in regulation of prothymosin α. Transient transfection assays were performed as before with a number of mutant alleles of c-*myc* that affect different domains of the protein. Control experiments show that all mutants are expressed at equivalent levels in these transient transfection assays (Fig. 7B). The data (Fig. 7A) show that the integrity of both the helix-loop-helix and leucine zipper domains is critical for activation of prothymosin α. All mutants in either of these domains abolish transactivation of prothymosin α by c-*myc*; in vitro immunoprecipitation assays show that all of these mutants also abolish the association of Myc with Max (48b). Second, the data show that an amino-terminal domain previously shown to be critical for transformation (Δ104-136) is also critical for transactivation; in contrast, smaller deletions within the amino terminus affected transactivation only moderately.

DISCUSSION

In this report, we show that regulation of prothymosin α by c-*myc* occurs via an E box localized within the first intron of the prothymosin α gene. c-*myc* had been suggested to regulate genes via E boxes mainly because of its ability to bind to these sequences in vitro and to transactivate synthetic reporter plasmids that carry E boxes in front of a minimal promoter (see the introduction). Evidence that c-*myc* regulates genes via such sequences in vivo is still limiting. The prothymosin α gene had been isolated as a c-*myc*-inducible gene by subtractive hybridization from a cell line that carries a hormone-inducible allele of c-*myc* (25). Our finding that the *cis*-responsive element in the prothymosin α gene contains a consensus E-box (CACGTG) sequence provides strong support that c-*myc* indeed activates genes via this sequence in vivo. Similar conclusions have recently been drawn from the analysis of the ornithine decarboxylase (7, 62), p53 (52), and ECA39 (8) genes, other genes that have been suggested to be regulated by c-*myc*.

Transcriptional activation by c-*myc* has been shown to depend on heterodimerization with Max (3, 41); it is not clear, however, whether Max is the only partner protein of Myc. Transcription of ornithine decarboxylase has been suggested to be activated by Myc in association with a partner protein other than Max (7). Support for this notion came from the finding that mutants of Myc that are unable to associate with Max were still capable of activating transcription of the ornithine decarboxylase gene. In contrast, all mutants of Myc that failed to associate with Max (In412, Δ370-412, and Δ412-434) (48a) also failed to transactivate transcription of prothymosin α (Fig. 7). This finding suggests that prothymosin α is activated by a Myc/Max complex. Consistent with this idea is our recent finding that MycER proteins associate with Max in a hormone-dependent manner (48c), providing a mechanistic model for how transcription of prothymosin α is induced by hormone in cells that carry MycER chimeras. However, it has been suggested that placing the *cis*-responsive element (which is found downstream of the transcription site in the ornithine decarboxylase, prothymosin α, p53, and ECA39 genes) in front of a minimal promoter may affect the outcome of the mutant analysis that we performed (14a). Further work is necessary to resolve this issue.

Many cell lines express a number of proteins that can bind to E-box sequences with a sequence specificity very similar or identical to that of Myc (10); these include USF (32), TFE-3 (6), and AP-4 (35). In nuclear extracts from RAT1A cells, USF is the predominant binding protein when a gel shift experiment is performed with the prothymosin α E box (56a), raising the question of why prothymosin α responds to activation of Myc in vivo. It is possible that although proteins other than Myc can bind to this sequence, they fail to activate after binding. Similar observations have been published for the achaete-scute protein, which binds to E boxes found in many muscle-specific genes but fails to activate transcription of these genes (19). Alternatively, binding of Myc to and/or transactivation by Myc from this sequence in vivo could be favored via protein-protein interactions with other factors binding to the prothymosin α

intron, possibly close to the CACGTG element. Support for this idea comes from the finding that synthetic reporter constructs that carry E boxes in front of a minimal promoter (and that would presumably lack such additional interactions) are, in contrast to prothymosin α, not activated by MycER in vivo (56a). Also, the E box that we have identified is not the only CACGTG element present in the prothymosin α gene; the promoter also contains such an element but did not respond to Myc in either transient or stable assays. Sequence comparison with the c-*myc*-responsive region of the ornithine decarboxylase gene reveals that the homology between the two elements extends significantly beyond the E box (Fig. 8), suggesting that factors other than Myc or Max might bind in the vicinity of and affect proteins binding to the E boxes. The availability of a Myc response element from an in vivo target gene of c-*myc* provides a useful model with which to address this question.

ACKNOWLEDGMENTS

This work was supported by the Bundesministerium für Forschung und Technologie (grant BCT0381-5) and the Human Frontier Science Program.

We thank David Schatz and Günther Schütz for pSV_2hygro and RSV-βgal, respectively.

REFERENCES

1. **Abrams, H. D., L. R. Rohrschneider, and R. N. Eisenman.** 1982. Nuclear location of the putative transforming protein of avian myelocytomatosis virus. Cell **29:**427–439.
2. **Amati, B., M. W. Brooks, N. Levy, T. D. Littlewood, G. I. Evan, and H. Land.** 1993. Oncogenic activity of the c-Myc protein requires dimerization with Max. Cell **72:**233–245.
3. **Amati, B., S. Dalton, M. W. Brooks, T. D. Littlewood, G. I. Evan, and H. Land.** 1992. Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. Nature (London) **359:**423–426.
4. **Amin, C., A. J. Wagner, and N. Hay.** 1993. Sequence-specific transcriptional activation by Myc and repression by Max. Mol. Cell. Biol. **13:**383–390.
5. **Ayer, D. E., L. Kretzner, and R. N. Eisenman.** 1993. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell **72:**211–222.
6. **Beckmann, H., L.-K. Suo, and T. Kadesh.** 1990. TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer μE3 motif. Genes Dev. **4:**167–179.
7. **Bello-Fernandez, C., G. Packham, and J. L. Cleveland.** 1993. The ornithine decarboxylase gene is a transcriptional target of c-MYC. Proc. Natl. Acad. Sci. USA **90:**7804–7808.
8. **Benvenisty, N., A. Leder, A. Kuo, and P. Leder.** 1992. An embryonically expressed gene is a target for c-Myc regulation via the c-Myc-binding sequence. Genes Dev. **6:**2513–2524.
9. **Berberich, S., D. N. Hyde, P. Espenshade, and M. Cole.** 1992. max encodes a sequence-specific DNA-binding protein and is not regulated by serum growth factors. Oncogene **7:**775–779.
10. **Blackwell, T. K., J. Huang, A. Ma, L. Kretzner, F. W. Alt, R. N. Eisenman, and H. Weintraub.** 1993. Binding of Myc proteins to canonical and noncanonical DNA sequences. Mol. Cell. Biol. **13:**5216–5224.
11. **Blackwell, T. K., L. Kretzner, E. M. Blackwood, R. N. Eisenman, and H. Weintraub.** 1990. Sequence-specific DNA binding by the c-myc protein. Science **250:**1149–1151.
12. **Blackwood, E. M., and R. N. Eisenman.** 1991. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with myc. Science **251:**1211–1217.
13. **Blackwood, E. M., B. Lüscher, and R. N. Eisenman.** 1992. Myc and Max associate in vivo. Genes Dev. **6:**71–80.
14. **Bustelo, X. R., A. Otero, J. Gómez-Márquez, and M. Freire.** 1991. Expression of the rat prothymosin alpha gene during T-lymphocyte proliferation and liver regeneration. J. Biol. Chem. **266:**1443–1447.

14a. **Cleveland, J.** Personal communication.

15. **Clinton, M., L. Graeve, H. El-Dorry, E. Rodriguez-Boulan, and B. L. Horecker.** 1991. Evidence for nuclear targeting of prothymosin and parathymosin synthesized *in situ*. Proc. Natl. Acad. Sci. USA **88:**6608–6612.
16. **Colby, W. W., E. Y. Chen, D. H. Smith, and A. D. Levinson.** 1983. Identification and nucleotide sequence of a human locus homologous to the v-*myc* oncogene of avian myelocytomatosis virus MC29. Nature (London) **301:**722–725.
17. **Conteas, C. N., M. G. Mutchnik, K. C. Palmer, F. E. Weller, G. D. Luk, P. H. Naylor, M. R. Erdos, A. L. Goldstein, C. Panneerselvam, and B. C. Horecker.** 1990. Cellular levels of thymosin immunoreactive peptides are linked to proliferative events: evidence for a nuclear site of action. Proc. Natl. Acad. Sci. USA **87:**3269–3273.
18. **Dang, C. V., M. McGuire, M. Buckmire, and W. M. F. Lee.** 1989. Involvement of the "leucine zipper" region in the oligomerization and transforming activity of human c-myc protein. Nature (London) **337:**664–666.
19. **Davis, R. L., P.-F. Cheng, A. B. Lassar, and H. Weintraub.** 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell **60:**733–746.
20. **Deuschle, U., R. Pepperkok, F. B. Wang, T. J. Giordano, W. T. McAllister, W. Ansorge, and H. Bujard.** 1989. Regulated expression of foreign genes in mammalian cells under the control of coliphage T3 RNA polymerase and lac repressor. Proc. Natl. Acad. Sci. USA **86:**5400–5404.
21. **Donner, P., I. Greiser-Wilke, and K. Moelling.** 1982. Nuclear localization and DNA binding of the transforming gene product of avian myelocatomatosis virus. Nature (London) **296:**262–266.
22. **Dosil, M., L. Alvarez-Fernandez, and J. Gómez-Márquez.** 1993. Differentiation-linked expression of prothymosin alpha gene in human myeloid leukemic cells. Exp. Cell Res. **204:**94–101.
23. **Downs, K. M., G. R. Martin, and J. M. Bishop.** 1989. Contrasting patterns of c-myc and N-myc expression during gastrulation of the mouse embryo. Genes Dev. **3:**860–869.

23a. **Eilers, M.** Unpublished data.

24. **Eilers, M., D. Picard, K. Yamamoto, and J. M. Bishop.** 1989. Chimaeras between the MYC oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature (London) **340:**66–68.
25. **Eilers, M., S. Schirm, and J. M. Bishop.** 1991. The MYC protein activates transcription of the alpha-prothymosin gene. EMBO J. **10:**133–141.
26. **Eschenfeldt, W. H., and S. L. Berger.** 1986. The human prothymosin α gene is polymorphic and induced upon growth stimulation: evidence using a cloned cDNA. Proc. Natl. Acad. Sci. USA **83:**9403–9407.
27. **Eschenfeldt, W. H., R. E. Manrow, M. S. Krug, and S. L. Berger.** 1989. Isolation and partial sequencing of the human prothymosin alpha gene family. J. Biol. Chem. **264:**7546–7555.
28. **Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop.** 1985. Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. Mol. Cell. Biol. **5:**3610–3616.
29. **Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn, and D. C. Hancock.** 1992. Induction of apoptosis in fibroblasts by c-myc protein. Cell **69:**119–128.
30. **Gómez-Márquez, J., and F. Sega.** 1988. Prothymosin α is a nuclear protein. FEBS Lett. **226:**217–219.
31. **Gómez-Márquez, J., F. Segade, M. Dosil, J. G. Pichel, X. R. Bustelo, and M. Freire.** 1989. The expression of prothymosin α gene in T lymphocytes and leukemic lymphoid cells is tied to lymphocyte proliferation. J. Biol. Chem. **264:**8451–8454.
32. **Gregor, P. D., M. Sawadogo, and R. G. Roeder.** 1990. The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes Dev. **4:**1730–1740.
33. **Gu, W., K. Cechova, V. Tassi, and R. Dalla-Favera.** 1993. Opposite regulation of gene transcription and cell proliferation by c-Myc and Max. Proc. Natl. Acad. Sci. USA **90:**2935–2939.
34. **Hirning, U., P. Schmid, W. A. Schulz, G. Rettenberger, and H. Hameister.** 1991. A comparative analysis of N-myc and c-myc

expression and cellular proliferation in mouse organogenesis. Mech. Dev. **33:**119–125.
35. **Hu, Y.-F., B. Luescher, N. Admon, N. Mermod, and R. Tjian.** 1990. Transcription factor AP-4 contains multiple dimerization domains that regulate dimer specificity. Genes Dev. **4:**1741–1752.
36. **Jansen-Dürr, P., A. Meichle, P. Steiner, M. Pagano, K. Finke, J. Botz, J. Wessbecher, G. Draetta, and M. Eilers.** 1993. Differential modulation of cyclin gene expression by *MYC*. Proc. Natl. Acad. Sci. USA **90:**3685–3689.
37. **Kato, G., W. M. F. Lee, L. Chen, and C. V. Dang.** 1992. Max: functional domains and interaction with c-myc. Genes Dev. **6:**81–92.
38. **Katz, S., E. Kowenz-Leutz, C. Müller, K. Meese, S. A. Ness, and A. Leutz.** 1993. The NF-M transcription factor is related to C/EBP β and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells. EMBO J. **12:**1321–1332.
39. **Keath, E. J., P. G. Caimi, and M. D. Cole.** 1984. Fibroblast lines expressing activated c-myc oncogenes are tumorigenic in nude mice and syngeneic animals. Cell **39:**339–348.
40. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35:**603–610.
41. **Kretzner, L., E. M. Blackwood, and R. N. Eisenmann.** 1992. Myc and Max proteins possess distinct transcriptional activities. Nature (London) **359:**426–429.
42. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240:**1759–1764.
43. **Manrow, R. E., A. Leone, M. S. Krug, W. H. Eschenfeldt, and S. L. Berger.** 1992. The human prothymosin alpha gene family contains several processed pseudogenes lacking deleterious lesions. Genomics **13:**319–331.
44. **Manrow, R. E., A. R. Sburlati, J. A. Hanover, and S. L. Berger.** 1991. Nuclear targeting of prothymosin alpha. J. Biol. Chem. **266:**3916–3924.
45. **Moses, H. L., E. Y. Yang, and J. A. Pietenpol.** 1990. TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. Cell **63:**245–247.
46. **Murre, C., P. SchonleberMcCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell **56:**777–783.
47. **Nordeen, S. K.** 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques **6:**454–457.
48. **Persson, H., and P. Leder.** 1984. Nuclear localization and DNA binding properties of a protein expressed by human c-myc oncogene. Science **225:**718–721.
48a. **Philipp, A.** Unpublished data.
48b. **Philipp, A., and M. Eilers.** Unpublished data.
48c. **Philipp, A., A. Schneider, I. Väsrik, K. Finke, Y. Xiong, D. Beach, K. Alitalo, and M. Eilers.** 1994. Repression of cyclin D1: a novel function of MYC. Mol. Cell. Biol. **14:**4032–4043.
49. **Prendergast, G. C., D. Lawe, and E. B. Ziff.** 1991. Association of myn, the murine homolog of max, with c-myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell **65:**395–407.
50. **Prendergast, G. C., and E. B. Ziff.** 1991. Methylation-sensitive sequence-specific DNA binding by the c-myc basic region. Science **251:**186–189.
51. **Reddy, C. D., P. Dasgupta, P. Saikumar, H. Dudek, F. J. Rauscher III, and E. P. Reddy.** 1992. Mutational analysis of Max: role of basic, helix-loop-helix/leucine zipper domains in DNA binding, dimerization and regulation of Myc-mediated transcriptional activation. Oncogene **7:**2085–2092.
52. **Reisman, D., N. B. Elkind, B. Roy, J. Beamon, and V. Rotter.** 1993. c-Myc trans-activates the p53 promoter through a required downstream CACGTG motif. Cell Growth Differ. **4:**57–65.
53. **Roy, A. L., C. Carruthers, T. Gutjahr, and R. G. Roeder.** 1993. Direct role for Myc in transcription initiation mediated by interactions with TFII-I. Nature (London) **365:**359–361.
54. **Roy, A. L., M. Meisterernst, P. Pornonec, and R. G. Roeder.** 1991. Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. Nature (London) **354:**245–248.
55. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
56. **Sburlati, A. R., R. E. Manrow, and S. L. Berger.** 1991. Prothymosin alpha antisense oligomers inhibit myeloma cell division. Proc. Natl. Acad. Sci. USA **88:**253–257.
56a. **Schneider, A.** Unpublished data.
57. **Shrivastava, A., S. Saleque, G. V. Kalpana, S. Artandi, S. P. Goff, and K. Calame.** 1993. Inhibition of transcriptional regulator Yin-Yang-1 by association with c-Myc. Science **262:**1889–1891.
58. **Smith, M. R., A. Al-Katib, R. Mohammad, A. Silverman, P. Szabo, S. Khilnani, W. Kohler, R. Nath, and M. G. Mutchnik.** 1993. Prothymosin α gene expression correlates with proliferation, not differentiation, of HL-60 cells. Blood **82:**1127–1132.
59. **Stone, J., T. de Lange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. Varmus, and B. Lee.** 1987. Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. Mol. Cell. Biol. **7:**1697–1709.
60. **Szabo, P., D. Ehleiter, E. Whittington, and M. E. Weksler.** 1992. Prothymosin alpha expression occurs during G1 in proliferating B or T lymphocytes. Biochem. Biophys. Res. Commun. **185:**953–959.
61. **Szabo, P., C. Panneerselvam, M. Clinton, M. Frangou-Lazaridis, D. Weksler, E. Whittington, M. J. Macera, K. H. Grzeschik, A. Selvakumar, and B. L. Horecker.** 1993. Prothymosin alpha gene in humans: organization of its promoter region and localization to chromosome 2. Hum. Genet. **90:**629–634.
62. **Wagner, A. J., C. Meyers, L. A. Laimins, and N. Hay.** 1993. c-myc induces the expression and activity ornithine decarboxylase. Cell Growth Differ. **4:**879–883.
63. **Watson, D. K., E. P. Reddy, P. H. Duesberg, and T. S. Papas.** 1983. Nucleotide sequence analysis of the chicken c-*myc* gene reveals homologous and unique coding regions by comparison with the transforming gene of avian myelocytomatosis virus MC29, Δ*gag-myc*. Proc. Natl. Acad. Sci. USA **80:**2146–2150.
64. **Watts, J. D., P. D. Cary, and C. Crane-Robinson.** 1989. Prothymosin α is a nuclear protein. FEBS Lett. **245:**17–20.
65. **Wenzel, A., C. Cziepluch, U. Hamann, J. Schurmann, and M. Schwab.** 1991. The N-Myc oncoprotein is associated in vivo with the phosphoprotein Max(p20/22) in human neuroblastoma cells. EMBO J. **10:**3703–3712.
66. **Westin, E. H., R. C. Gallo, S. K. Arya, A. Eva, L. M. Souza, M. A. Baluda, S. A. Aaronson, and F. Wong-Staal.** 1982. Differential expression of the *amv* gene in human hematopoietic cells. Proc. Natl. Acad. Sci. USA **79:**2194–2198.
67. **Westin, E. H., F. Wong-Staal, E. P. Gelmann, R. Dalla-Favera, T. S. Papas, J. A. Lautenberger, A. Eva, E. P. Reddy, S. R. Tronik, S. A. Aaronson, and R. C. Gallo.** 1982. Expression of cellular homologues of retroviral *onc* genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA **79:**2490–2494.
68. **Zalvide, J. B., E. Cancio, C. V. Alvarez, B. J. Regueiro, and F. Dominguez.** 1992. Prothymosin α mRNA levels are invariant throughout the cell cycle. J. Biol. Chem. **267:**8692–8695.
69. **Zervos, A. S., J. Gyuris, and R. Brent.** 1993. Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell **72:**223–232.