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An E-Box-Mediated Increase in *cad* Transcription at the G_1/S-Phase Boundary Is Suppressed by Inhibitory c-Myc Mutants

ROSALYNN J. MILTENBERGER, KRISTINE A. SUKOW, AND PEGGY J. FARNHAM*

McArdle Laboratory for Cancer Research, University of Wisconsin—Madison Medical School, Madison, Wisconsin 53706

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To better understand the signaling pathways which lead to DNA synthesis in mammalian cells, we have studied the transcriptional activation of genes needed during the S phase of the cell cycle. Transcription of the gene encoding a pyrimidine biosynthetic enzyme, carbamoyl-phosphate synthase (glutamine-hydrolyzing)/aspartate carbamoyltransferase/dihydroorotase (*cad*), increases at the G_1/S-phase boundary. We have mapped the growth-dependent response element in the hamster *cad* gene to the extended palindromic E-box sequence, CCACGTGG, which is centered at +65 in the 5′ untranslated sequence. Mutation of the E box abolished growth-dependent transcription, and an oligonucleotide corresponding to the *cad* sequence at +55 to +75 (+55/+75) restored growth-dependent regulation to nonresponsive *cad* promoter mutants when placed downstream of the transcription start site. The same oligonucleotide conferred less G_1/S-phase induction when placed upstream of basal promoter elements. An analogous oligonucleotide containing the mutant E box had no effect in either location. Nuclear proteins bound the *cad* +55/+75 element in a cell cycle-dependent manner in electromobility shift assays; antibodies specific to USF and Max blocked the DNA-binding activity of different growth-regulated protein-DNA complexes. Expression of c-Myc mutants which have been shown to dominantly interfere with the function of c-Myc and Max significantly inhibited *cad* transcription during S phase but had no effect on transcription from another G_1/S-phase-activated promoter, *dhfr*. These data support a model whereby E-box-binding proteins activate serum-induced transcription from the *cad* promoter at the G_1/S-phase boundary and suggest that a Max-associated protein complex contributes to the serum response.

Cellular responses to external growth stimuli involve complex signal transduction pathways which lead to changes in the expression of genes needed for DNA synthesis and cell division. The carbamoyl-phosphate synthase (glutamine-hydrolyzing)/aspartate carbamoyltransferase/dihydroorotase (*cad*) gene encodes a trifunctional enzyme which catalyzes the first three steps in the de novo synthesis of pyrimidines (21). In mammalian cells, the levels of both CAD enzymatic activity and *cad* mRNA correlate with the proliferative state of the cell. For example, the endogenous *cad* mRNA level increased approximately 10-fold at the G_1/S transition following serum stimulation of quiescent cells (58). Nuclear run-on assays demonstrate a 3- to 10-fold increase in the rate of *cad* transcription after serum stimulation, suggesting that a significant portion of growth-dependent regulation occurs at the transcriptional level (44, 58). In accordance with a model of transcriptional regulation, the activity of a 164-bp region of the *cad* promoter fused to a reporter gene increases 15-fold at the G_1/S-phase boundary in a serum starvation/stimulation assay (50). To identify the factor(s) which coordinates *cad* expression with the growth responsiveness of the cell, we have analyzed the requirements for accurate *cad* transcription in cultured cells and in cell extracts.

The sequences of the Syrian hamster *cad* promoter that support growth-responsive transcription extend from positions −81 to +83 (50), relative to the major start site of transcription (22). In a footprinting assay with HeLa nuclear extract, three major regions of the −81/+83 promoter are protected from DNase I cleavage (22). The first region binds the transcription factor Sp1 at two sites, one near −70 and the other near −49 (22, 34). Sp1 binding is critical for basal promoter activity, and the proximal Sp1 site positions the start site of transcription from this TATA-less promoter (35). The second major protected region binds a ubiquitous activator protein, designated Honk, at −17 (35). Preliminary results suggest that neither the Sp1 sites nor the Honk site play a role in growth-dependent regulation; that is, promoter constructs mutated at these sites still display substantial increases in *cad* transcription at the G_1/S-phase boundary (49a). A third region of the *cad* promoter that binds protein in a HeLa footprinting assay spans the sequence from +55 to +75. In the center of these 21 nucleotides lies a palindromic sequence which matches the binding consensus for E-box-binding proteins such as USF (7), c-Myc, and Max (63). Immediately adjacent to the protected region, between +75 and +83, lies an E2F-like site in which 7 of 8 bases match the E2F-binding consensus (51, 53).

USF, c-Myc, Max, and the E2F family of proteins share a common structural feature, the helix-loop-helix domain, which mediates dimerization between functional protein partners. The E2F gene family of transcription factors includes E2F1, E2F2, E2F3, E2F4, DP1, and DP2. On the basis of cooperativity in DNA-binding and transactivation assays, it is believed that E2F1 (or E2F2, E2F3, or E2F4) heterodimerizes with DP1 (or DP2) to form E2F activity at the DNA sequence TTTSSCGC (where S is C or G) in viral and cellular genes (4, 27, 30, 36). Examples of E2F-regulated genes include the dihydrofolate reductase gene (*dhfr*) (49, 62), the thymidine kinase gene (17, 42), and B-*myb* (38, 39), all of which are induced at the G_1/S-phase boundary. The family of proteins which recognize a hexanucleotide sequence known as an E box (CANNTG, where N is A, C, G, or T) include the adenovirus major late transcription factors USF/MLTF (24), TFE3 (5), TFEB (13), and AP-4 (29); the heterodimeric transcriptional activator c-Myc:Max and the homodimeric transcriptional re-

* Corresponding author. Mailing address: McArdle Laboratory for Cancer Research, University of Wisconsin—Madison Medical School, 1400 University Ave., Madison, WI 53706. Phone: (608) 262-2071. Fax: (608) 262-2824.

pressor Max:Max (1, 10, 37, 55); and the transactivation-incompetent, differentiation-specific complexes Mad:Max (2, 3) and Mxi1:Max (67).

An abundance of evidence has amassed in recent years implicating members of the E2F and E-box families, specifically E2F1 and c-Myc, as key regulators of proliferation and growth cycle progression (for reviews, see references 20, 23, 46, and 48). Both contribute to the transformation of primary rat embryo fibroblasts when overexpressed in culture (40, 61). Both are down-regulated upon growth arrest (15, 62, 65), and in some cell types, inducing expression of c-Myc (18) or E2F1 (31) ectopically in quiescent cells is sufficient to cause entry into the S phase. Conversely, inhibiting c-*myc* expression via antisense oligonucleotides or antisense transcripts prevents mitogen-treated cells from entering the S phase, providing further evidence that c-Myc plays an important role in progression through the G_1 phase of the growth cycle (26, 56). In two model systems in which the growth cycle has been well characterized, serum induction of quiescent cells (33, 57, 64) and partial hepatectomy (47), c-Myc expression increases prior to the G_1 restriction point and returns to a somewhat lower level that remains invariant through the rest of the cycle. Levels of E2F1 increase later at the G_1/S-phase boundary (28, 62), which is consistent with the role of this protein as a direct activator of genes, such as *dhfr*, that are needed for DNA synthesis. A few potential c-Myc target genes have been identified, including the α-prothymosin gene (18), *ECA39* (8), *p53* (59), and the ornithine decarboxylase gene (*odc*) (6). Some of these c-Myc-activated genes are associated with cellular proliferation but cannot be placed into a single category on the basis of their temporal pattern of expression through the cell growth cycle.

To more precisely delineate the factors which regulate *cad* expression, we investigated the potential role of both the E box and putative E2F site in activating G_1/S-phase transcription from the *cad* promoter. Here, we show that the E box is both necessary and sufficient for growth-dependent expression of *cad* and that the E2F-like site is dispensable. By use of both in vitro and in vivo analyses, we address the potential contribution of USF, c-Myc, and Max to *cad* transcription at the G_1/S-phase boundary.

MATERIALS AND METHODS

Plasmids. Standard cloning techniques were used for all plasmid constructions (60). *cad* reporter plasmids contain promoter fragments cloned upstream of the luciferase cDNA in the vector pGL2Basic (Promega). Construction of cad−81/+83 and cad−81/+26 has been described previously (35). The construct cad−81/+55 was created by blunt-end ligation of *Esp*I-*Hin*dIII-digested cad−81/+83. The construct cadE2Fmt was created by inserting a double-stranded oligonucleotide which contains a blunt 5′ end, a mutation at the E2F-like site, and a 3′ *Hin*dIII cohesive end into the *Pml*I-*Hin*dIII sites of cad−81/+83. The sequence of the top strand of the oligonucleotide reads 5′-GTGGACCAA**CCCCTATAT**G gggatctaagta-3′, with wild-type *cad* promoter sequences indicated by capital letters, mutant bases indicated by boldface type, and vector sequences indicated by lowercase letters. The construct cadEboxmt was generated from cad−81/+83 by PCR with pGLprimer1 (Promega) to prime synthesis of the coding (top) strand and a 56-base oligonucleotide spanning from *cad* +50 to 5 bases beyond the *Hin*dIII site in pGL2Basic to prime synthesis of the noncoding (bottom) strand. The latter primer introduced an E-box mutation (CCACGTGG to **ACTGCA**GG). The amplified fragment was inserted into the *Kpn*I-*Hin*dIII sites of pGL2Basic. The constructs cad+26/Ebox and cad+26/Emt were created by inserting a double-stranded oligonucleotide which corresponds to *cad* promoter sequences +55/+75 and contains filled-in *Bgl*II ends into the filled-in *Hin*dIII site of cad−81/+26. The top strands of the wild-type and mutant oligonucleotides read as follows:

5′-gatctAGCGAGCCACGTGGACCAACTa-3′ (wild type, Ebox)

5′-gatctAGCGAG**ACTGCA**GGACCAACTa-3′ (mutant, Emt)

The same blunt-ended oligonucleotides were inserted into the *Sma*I site of cad−81/+26 to create Ebox/cad+26 and Emt/cad+26. The *dhfr* reporter plasmid pWTluc has been described previously (49).

The TI construct contains a TATA box and an initiator element cloned into pGL2Basic (50). The construct STI was created by inserting *cad* promoter sequences −75/−43, which contain two consensus Sp1-binding sites (22, 34), into the *Sma*I site of TI. To create S/Ebox/TI, the +55/+75 (Ebox) oligonucleotide was inserted into the *Bgl*II site of the plasmid STI. The STI/Ebox plasmid was created by inserting the same oligonucleotide, which was first blunt ended with Klenow fragment, into the *Hin*dIII site of plasmid STI. Each reporter construct was verified by sequencing.

The mouse sarcoma virus long terminal repeat (LTR) drives expression of wild-type and mutant human c-*myc* cDNAs which were cloned into the *Eco*RI site of a plasmid originally derived from a Bluescript (Stratagene) vector (37). The LTR construct expresses no cDNA. The LTR-MycΔBr construct expresses a derivative of human c-Myc lacking amino acids 353 to 367 in the basic DNA-binding domain (10). The LTR-MycΔTAD construct expresses a human c-Myc mutant lacking amino acids 44 to 170 in the transactivation domain. It was created by removing a 413-bp *Pst*I fragment from the wild-type human c-*myc* cDNA in plasmid LTR-Myc. Numbering refers to the amino acid sequence of human c-*myc* (66).

Cell culture and transfections. NIH 3T3 cell cultures were maintained as described previously (50). One day prior to transfection, 1.5×10^5 to 2×10^5 cells were seeded into 60-mm dishes. For time course experiments, each plate of cells was transfected with 1 μg of *cad* reporter DNA and 14 μg of sonicated salmon sperm DNA or with 3 μg of TI reporter DNA and 12 μg of sonicated salmon sperm DNA. For cotransfections, cells received 1 μg of reporter DNA, 3 μg of expression plasmid, and 11 μg of sonicated salmon sperm DNA. Cells were transfected by the calcium phosphate method (49) for 6 h and then subjected to by glycerol shock (50). Cells were then growth arrested by replacing the maintenance medium (5% serum) with starvation medium (0.4% serum). After 2 days, the cells were stimulated to reenter the proliferative cell cycle by replacing starvation medium with stimulation medium (10% serum). Cells were harvested at specified times following serum stimulation and assayed for luciferase activity (50). For 22-h time courses, cells were harvested at 2-h intervals following stimulation. For monitoring promoter activity during S phase only, cells were harvested at 0, 12, 14, and 16 h following serum stimulation. Transfection efficiencies typically ranged from 2 to 5% as determined by β-galactosidase assays (45). Growth cycle progression was monitored by flow-cytometric analysis of propidium iodide-stained cells (62). Each transfection was repeated at least twice with duplicate samples and multiple DNA preparations.

Nuclear extract preparation. Nuclear extracts were prepared from NIH 3T3 cells, after serum starvation (0 h) and stimulation for 4, 8, or 12 h (3×10^7 cells per time point), by a modification of a published method (16). Cells were swollen in 500 μl of RSB hypotonic buffer (19) supplemented with 0.5 mM dithiothreitol, 100 μg of phenylmethylsulfonyl fluoride (PMSF) per ml, 1 μg of orthovanadate per ml, and 10 μg of leupeptin per ml. The swollen cells were supplemented with 0.5% (vol/vol) Nonidet P-40, and the nuclei were released by Dounce homogenization. Nuclei were pelleted at 4,000 × *g* for 5 min and washed briefly with RSB to remove residual Nonidet P-40. Nuclear proteins were extracted at 4°C for 30 min in 100 μl of buffer C (16) supplemented with 0.5% deoxycholate, 1.0% octyl-β-glucoside, 1 μg of orthovanadate per ml, and 10 μg of leupeptin per ml. After dialysis against buffer D (16) for 1 h at 4°C, nuclear extracts were quantitated by the Bradford assay (Bio-Rad) and stored at −70°C.

Electromobility shift assays. Electromobility shift assays were performed as described previously (41) with the following modifications. In a total volume of 18 μl, 6 to 12 μg of nuclear extract, 2 μg of sonicated salmon sperm DNA, 11 μl of binding buffer (7.1 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.0], 3.6 mM $MgCl_2$, 100 mM KCl, 5.7% glycerol, 0.03% Nonidet P-40), and specific competitor DNA (when indicated) were incubated together for 10 min at room temperature. When specified, antibodies were then added to individual reactions, and the mixtures were incubated for an additional 10 to 20 min at room temperature. Oligonucleotide probes (0.5 ng of DNA per reaction) which had been end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (60) were added to the reactions, and incubation was continued at room temperature for 10 to 20 min. The reaction mixtures were then electrophoresed for 90 min on a 4% polyacrylamide gel (29:1 acrylamide/bisacrylamide) which had been preelectrophoresed for 30 to 60 min. The gel and electrophoresis buffer was 22.5 mM Tris–morpholinepropanesulfonic acid (MOPS; pH 7.0)–0.5 mM EDTA. Gels were dried prior to autoradiography. The amount of antibody used in competition experiments was as follows: 1 μl of dilute (1:100 in binding buffer) anti-human USF antiserum from rabbits (gift from R. Roeder), or 2 μg of anti-human c-Myc antiserum from rabbit 1537-15 (gift from R. N. Eisenman; raised against the C terminus), anti-human Max antiserum from rabbit 8711A (gift from R. N. Eisenman; raised against the entire protein), anti-human c-Myc monoclonal antibody from mice (Santa Cruz Biotechnology), anti-human Max from rabbits (Santa Cruz Biotechnology), or normal rabbit serum pooled from three individual preimmunized rabbits (gift of the R. Burgess laboratory).

Western immunoblot analysis. Growth cycle-staged NIH 3T3 cells (6×10^6 cells at each time point) were swollen and lysed and the nuclei were isolated as described above. Nuclei were then resuspended in 200 μl of RSB containing 0.5% deoxycholate, 1.0% octyl-β-glucoside, 0.5 mM dithiothreitol, 100 μg of phenylmethylsulfonyl fluoride per ml 1 μg of orthovanadate per ml, and 10 μg of leupeptin per ml. The lysed nuclei were passed through a 22-gauge needle until they were no longer viscous, and they were stored at −70°C. One-third of each

sample (2×10^6 cells) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a 10% polyacrylamide gel (29:1 acrylamide/bisacrylamide) by standard techniques (60). Proteins were transferred to nitrocellulose and probed with anti-human Myc antiserum 1537-15 (gift from R. N. Eisenman) at 1:500 and then with a peroxidase-conjugated anti-rabbit secondary antibody from goats (Boehringer-Mannheim). Proteins were detected by enhanced chemiluminescence (Kirkegaard & Perry). The same blot was then probed with either anti-human USF antiserum (gift from R. Roeder) at 1:10,000 or anti-human Max antiserum 8711A (gift from R. N. Eisenman) at 1:5,000; this was followed by probing with the same secondary antibody as above and enhanced chemiluminescence detection.

RESULTS

Identification of the growth-responsive element in the *cad* promoter. To better localize the growth-responsive element within the −81/+83 promoter, we examined the activity of a *cad* construct whose 3′ terminus lay at +55 (cad−81/+55) in a growth response assay. The luciferase reporter construct was transfected into a growing population of cells, which were then serum starved to induce growth arrest. When stimulated with serum-rich medium, the cells synchronously progress through one growth cycle. Figure 1 shows that unlike the −81/+83 fragment, the −81/+55 region of the *cad* promoter does not confer growth-dependent regulation to the reporter gene. The truncated promoter provided at least as much basal transcriptional activity as did the wild-type promoter in quiescent cells, as a result of the presence of two intact Sp1 sites which have been shown previously to be essential for promoter activity in vitro and in vivo (35). In contrast to the cad−81/+83 construct that was activated 15-fold in more than three independent experiments, the −81/+55 promoter was activated only 2- to 3-fold at the G_1/S-phase boundary. These results indicate that the region downstream of +55 contains an element critical for growth responsiveness but dispensable for overall promoter activity.

Potential recognition sequences for two classes of transcription factors lie between *cad* promoter sequences +55 and +83. To examine the contribution of each sequence to the growth-responsiveness of *cad*, the E2F-like site at +75 and the E box at +65 were independently mutagenized within the context of the −81/+83 promoter background. The *cad* E2F-like site was changed to a sequence which abolished E2F binding to and growth-dependent regulation of the murine *dhfr* promoter (49). Similar to the −81/+83 promoter, the activity of the cadE2Fmt construct began to increase at 8 h following stimulation and climbed to a clear peak within S phase. Although the induction level in S phase was lower for the cadE2Fmt construct than it was for the wild-type construct, the overall shape of the two serum response curves was similar. In contrast, substituting 5 of 8 bases at the extended E-box sequence, CCACGTGG, abolished the characteristic rise in transcriptional activity that begins 8 h following serum stimulation. Induction of the cadEboxmt construct was nearly indistinguishable from that of the nonresponsive −81/+55 deletion mutant throughout G_1 phase and at the G_1/S-phase transition. The E-box mutation did not adversely affect basal promoter activity, however, since the absolute level of promoter activity in quiescent cells was at least as great as the activity of the wild-type construct. Comparison of the general shape of individual serum response curves established a clear difference between the serum inducibility of the cadEboxmt and cadE2Fmt promoters. These data implicate a more critical role for the E box than for the E2F-like sequences in mediating serum-induced transcription from the *cad* promoter. However, since the E2F mutation did significantly depress the level of induction during S phase, the possibility exists that E2F cooperatively interacts with the more critical regulatory factors at the adjacent E-box motif.

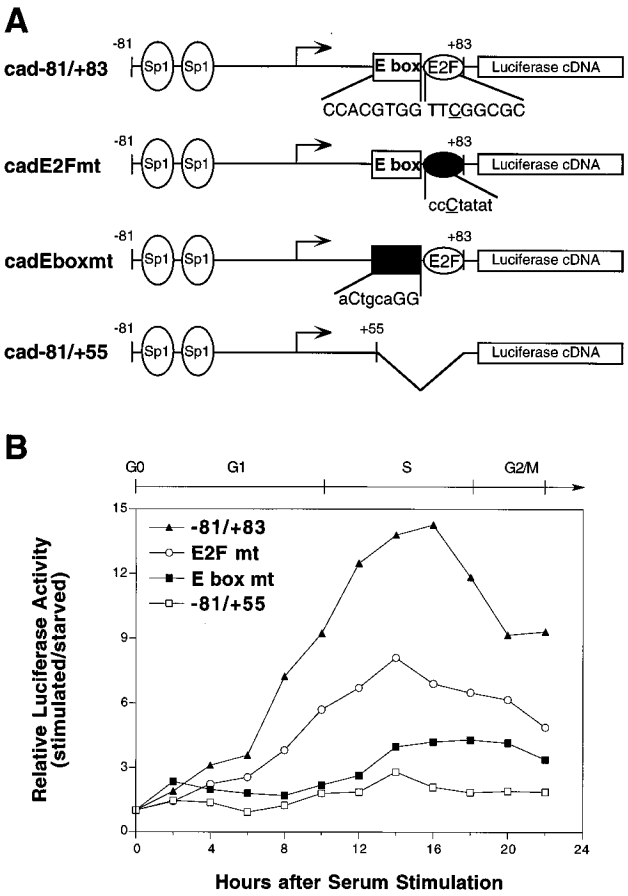

FIG. 1. Induction of growth-regulated transcription from wild-type and mutated versions of the *cad* promoter. (A) Schematic representation of *cad* reporter constructs. Hamster *cad* promoter sequences were fused to the luciferase cDNA, as indicated. Promoter coordinates are enumerated by the nucleotide distance from the major transcription start site (arrow). Relative locations of the Sp1-binding sites, the E box, and the E2F-like site (E2F) are approximated by open symbols. Solid symbols represent mutated sites. Wild-type and mutant sequences are indicated below the symbols by capital and lowercase letters, respectively. The underlined base in the E2F-like site represents a mismatch to the E2F-binding consensus. (B) Graphical representation of the average induction of *cad* reporter activity through the growth cycle of NIH 3T3 cells. Cells were transiently transfected with the indicated reporter constructs and serum starved or stimulated. Promoter induction is reported as the ratio of luciferase activity measured in cells harvested at the indicated times following serum stimulation relative to activity from the same construct in serum starved cells. The absolute level of luciferase activity measured in serum-starved cells was comparable for each construct within individual experiments and was 10- to 150-fold greater than the activity measured in mock-transfected cells. Data represent the average of 3 to 10 independent experiments. The standard error for each construct was greatest at the peak of induction. The percent standard error at the time of peak induction was 34% for cad−81/+83, 16% for cadE2Fmt, 40% for cadEboxmt, and 29% for cad−81/+55. The stages of the growth cycle are indicated above the graph and were determined by flow cytometry (data not shown).

The +55/+75 region of the *cad* promoter is sufficient to confer growth responsiveness to a nonresponsive, minimal *cad* promoter. The *cad* sequences from −81 to +26 constitute the minimal promoter region necessary and sufficient for accurate transcription initiation in vitro and in vivo (35). While these promoter sequences provide strong basal transcriptional activity, they do not support growth-dependent transcription (Fig. 2). To determine if the *cad* E-box region alone could confer growth responsiveness to this minimal *cad* promoter, a 21-bp

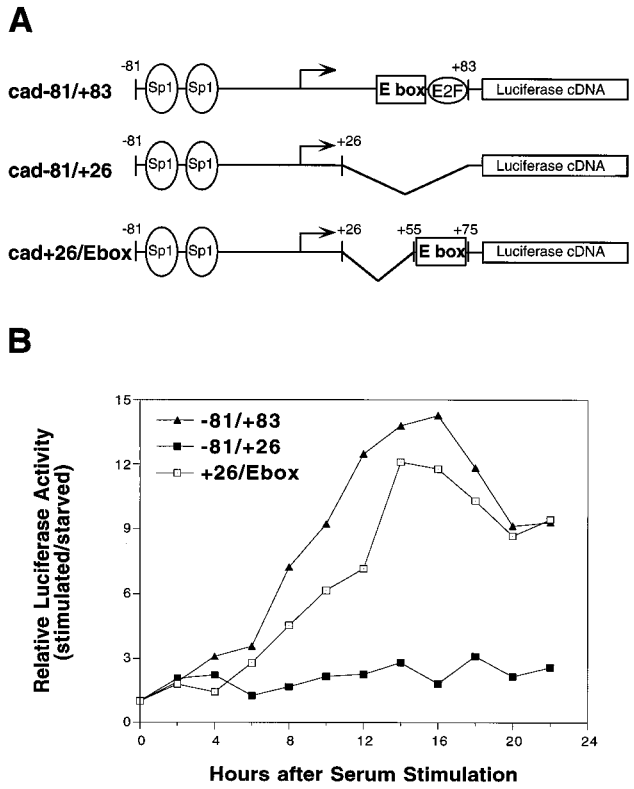

FIG. 2. Induction of growth-regulated transcription from a hybrid *cad* promoter containing basal elements and the E-box region. (A) Schematic representation of *cad* reporter constructs. (B) Graphical representation of the average induction of *cad* reporter activity through the growth cycle of NIH 3T3 cells. Cells were transiently transfected with the indicated reporter constructs and serum starved or stimulated as described in the legend to Fig. 1. Data represent the average of 2 to 10 independent time course experiments. The standard error for each construct was greatest at the peak of promoter activity. The percent standard error at the time of peak induction was 34% for cad−81/+83, 20% for cad−81/+26, and 46% for cad+26/Ebox.

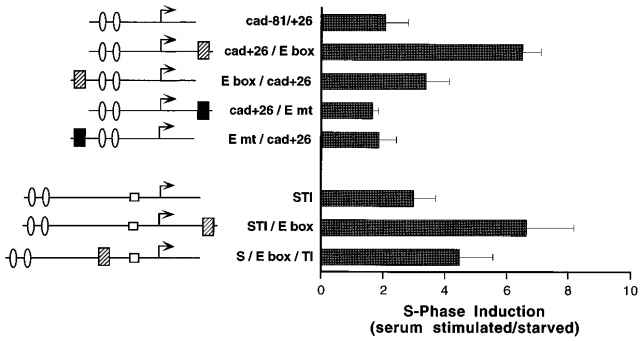

FIG. 3. Effect on S-phase induction when the *cad* E box is positioned upstream versus downstream of basal promoter elements. NIH 3T3 cells were transfected with the indicated reporter plasmids and serum starved or stimulated. Cells were harvested in duplicate at 0, 12, 14, and 16 h following serum stimulation. Luciferase activity was measured at each time point and averaged between duplicate samples. S-phase induction was determined by dividing the maximium promoter activity in serum-stimulated cells by the level in serum-starved cells. The absolute level of luciferase activity measured in serum-starved cells was 10- to 70-fold greater than the activity measured in mock-transfected cells. The maximum induction obtained in three to nine independent experiments was averaged and is represented in the figure. Error bars indicate the standard error between experiments. The Sp1 sites (open ovals), the wild-type E box (hatched box; core sequence, CCACGTGG), the mutant E box (solid box; core sequence, aCtgcaGG), the TATA box (open box), and the transcription start site (arrow) are indicated.

oligonucleotide (*cad* sequences +55/+75) that contains the E box but excludes the E2F-like site was inserted downstream of *cad* sequences −81/+26. In the resulting hybrid promoter (cad+26/Ebox), the +55/+75 region lies 36 bp downstream from the transcription start site, which is approximately 20 bp closer than in the cellular promoter. The cad+26/Ebox construct clearly exhibits nearly wild-type levels of regulated expression through the growth cycle (Fig. 2). An analogous hybrid promoter containing the +55/+75 region with a mutated E box (**ACTGCA**GG) was unresponsive at the G_1/S-phase boundary (Fig. 3), indicating that the E-box sequence CCA CGTGG is required for the growth-dependent increase in transcriptional activity. Likewise, the −81/+55 promoter was rendered fully serum inducible at the G_1/S-phase boundary by reintroduction of the E-box sequences but not the E2F-like sequences downstream (data not shown). Taken together with the deletion and mutagenesis studies above, these results indicate that the E box is both necessary and sufficient for increased transcription from the *cad* promoter at the G_1/S-phase boundary. Furthermore, since the +55/+75 element alone conferred growth responsiveness, the E2F-like site appears dispensable for growth-dependent regulation of *cad*. The diminished inducibility of the cadE2Fmt construct relative to the wild type may indicate that base substitution at the E2F-like site altered sequences flanking the E box to those unfavorable for binding by E-box-binding proteins.

Several groups have demonstrated that multiple copies of the CACGTG sequence placed 5′ to +1 will activate transcription in proliferating cells, but the activity of these E-box-driven promoters was not examined during the transition from quiescence to a proliferative state. To determine if the *cad* E box could confer G_1/S-phase activation when placed either upstream or downstream of the transcription start site, a variety of synthetic hybrid promoters were constructed (Fig. 3). The maximum transcriptional activity observed for each promoter during the S phase of the growth cycle was compared with promoter activity in serum-starved cells. As described in Fig. 2, the +55/+75 region of *cad* conferred considerable S-phase activation to the minimal *cad* promoter when it was inserted downstream of the transcription start site (cad+26/Ebox). In contrast, when the +55/+75 sequences were located upstream (Ebox/cad+26), approximately 20 bp 5′ to the Sp1 sites in the −81/+26 *cad* promoter, transcription from the resulting construct was induced about threefold during S phase, which was about half that observed when a single site was placed downstream. This modest level of induction is reminiscent of the gradual increase in activity from the simian virus 40 early promoter throughout the growth cycle (62). Again, mutant E-box sequences had no effect when placed either upstream (Emt/cad+26) or downstream (cad+26/Emt). Similar results were observed when pieces of the *cad* promoter were reassembled in the context of a heterologous, synthetic promoter which contains only two Sp1 sites, a TATA box, and an initiator region (STI). Placing the *cad* +55/+75 sequence downstream of the start site in the STI promoter (STI/Ebox) resulted in greater S-phase activity than placing it upstream of +1 (S/Ebox/TI) did. Despite the inherant caveat of potentially suboptimal spacing between the several transcription factor-binding sites, a general trend did emerge. The *cad* E-box region conferred greater transcriptional activity in S phase when placed in a transcribed region of the promoter, as opposed to upstream of +1.

Nuclear proteins bind *cad* +55/+75 sequences in a growth cycle-dependent manner. Previous footprinting analysis has es-

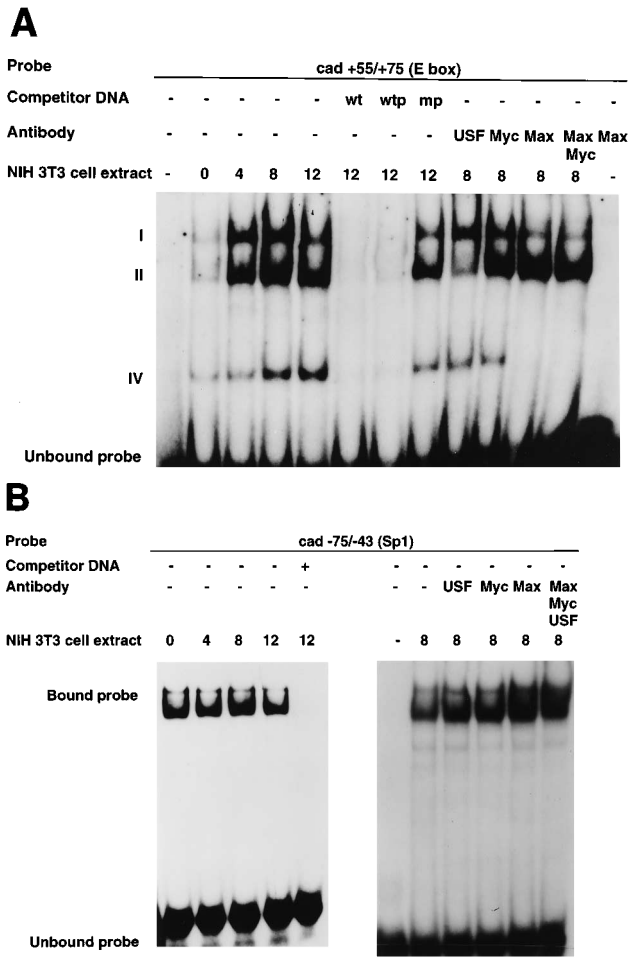

FIG. 4. Nuclear protein binding to *cad* promoter elements in growth cycle-staged extracts from NIH 3T3 cells. (A) Binding to the *cad* E-box region. End-labeled, double-stranded oligonucleotide probes corresponding to *cad* promoter sequences +55/+75 were incubated with no extract (−) or nuclear extract from NIH 3T3 cells harvested at 0, 4, 8, or 12 h following stimulation of serum-starved cells. The 12-h extract was preincubated with a 50-fold molar excess of competitor DNAs prior to the addition of probe: no DNA (−), unlabeled probe DNA (wt; core sequence, CCACGTGG), the wild-type −81/+83 *cad* promoter fragment (wtp; E-box core sequence, CCACGTGG), or the −81/+83 *cad* promoter fragment carrying a mutated E box (mp; E-box core sequence, a CtgcaGG). The 8-h extract was preincubated with either no antibody (−) or antisera specific to human USF, c-Myc, Max, or combinations thereof prior to the addition of probe. The DNA-protein complexes are labeled I, II, and IV; an additional complex, III, can be detected with K562 extract (data not shown). (B) Binding to the *cad* Sp1 sites. In the left panel, end-labeled double-stranded oligonucleotide probes corresponding to *cad* sequences −75/−43 were incubated with nuclear extract from NIH 3T3 cells harvested at 0, 4, 8, or 12 h following stimulation of serum-starved cells. The 12-h extract was preincubated with either no DNA (−) or a 50-fold molar excess of unlabeled probe DNA (+) prior to the addition of radiolabeled probe. In the right panel, the 8-h extract was either not preincubated (−) or preincubated with antisera specific to human USF, c-Myc (Myc), Max, or combinations thereof, as in panel A.

tablished that nuclear proteins bind the +55/+75 region of the *cad* promoter in proliferating HeLa cells (22). However, the identity of the DNA-bound proteins remained unknown. As a first step toward characterizing these proteins, we used growth cycle-staged extracts in an electrophoretic mobility shift assay to confirm that nuclear proteins could bind the +55/+75 region in NIH 3T3 cells. Figure 4A shows that three major protein complexes bound a radiolabeled +55/+75 probe in NIH 3T3 cells. A similar pattern of complex formation was observed in extracts from proliferating mouse Friend cells and human K562 cells, except that an additional complex (III) was observed when K562 extract was used (data not shown). Binding activity to the +55/+75 probe was barely detectable in quiescent NIH 3T3 cells and became more pronounced at 8 and 12 h following serum stimulation (Fig. 4A, leftmost five lanes), which closely correlates with levels of *cad* transcriptional activity. Complex formation was specifically inhibited by a 10- to 50-fold molar excess of unlabeled probe DNA or the entire −81/+83 *cad* promoter fragment but not by analogous competitors containing the E-box mutation which abolished growth-dependent regulation of the *cad* promoter (Fig. 4A, lanes labeled wt 12, wtp 12, and mp 12). To control for protein quantitation in each extract, Sp1-binding activity to *cad* sequences −75/−43 was assayed (Fig. 4B). As expected (43), Sp1-binding activity was invariant through the growth cycle of NIH 3T3 cells. These data indicate that the increased complex formation at *cad* sequences +55/+75 in staged extracts reflects an enhanced affinity of nuclear proteins for the *cad* E box as a function of the growth state of the cell.

To identify the proteins bound to the +55/+75 probe, we preincubated extracts with antibodies raised against specific E-box family members (Fig. 4A). In each case, inhibition of DNA-protein complexes was specific to proteins bound to the *cad* E-box probe, since the antisera did not interfere with Sp1-DNA complex formation (Fig. 4B, panel 2). Moreover, equal or greater amounts of preimmune serum had no effect on the E-box-bound complexes (data not shown), indicating that rabbit serum does not contain a general inhibitor of DNA-protein interactions. Antiserum specific for USF significantly blocked formation of complex II (Fig. 4A, lane labeled USF 8); similarly, anti-Max antibodies efficiently blocked formation of complex IV and reduced formation of complex I (Fig. 4A, lanes labeled Max 8 and Max Myc 8). Whether complex I or IV consists of Max homodimers or other Max partners cannot be ascertained from these data. Thus, our analysis confirmed that both USF and Max can recognize the *cad* E box, a result expected from consensus definitions for the DNA-binding specificities of these proteins in vitro (7, 63).

Although we could detect growth-dependent binding of Max and USF by electrophoretic mobility shift assay, we could not detect c-Myc in NIH 3T3 extracts. To ensure that c-Myc was expressed in our NIH 3T3 cells and to determine the relative abundance of USF, c-Myc, and Max proteins through the growth cycle, Western analysis was performed with whole nuclear lysates prepared by more stringent extraction procedures from serum-starved and -stimulated NIH 3T3 cells (Fig. 5). As expected, the levels of c-Myc protein were barely detectable in serum-starved NIH 3T3 cells (15, 33, 65); peak c-Myc expression occurred 8 h following serum stimulation, which is when *cad* transcription begins to increase in transfection experiments. USF was detectable at relatively constant levels in both quiescent and serum-stimulated cells. Max was also present throughout the growth cycle, as previously reported (9, 11; data not shown). Taken together with the results of the DNA-binding studies, these results indicate that the DNA-binding activities of Max and USF are regulated in a growth-dependent manner, despite their constitutive expression. Therefore, it is likely that posttranslational events increase the affinity of Max and USF for the *cad* E box during the transition from quiescence to a growing state. Finally, while c-Myc was not detectable in NIH 3T3 extracts used for examining DNA-binding activity, it was easily detectable in more stringently prepared lysates of NIH 3T3 nuclei. These observations indicate that recovery of c-Myc is sensitive to extraction procedures, as documented by others (19).

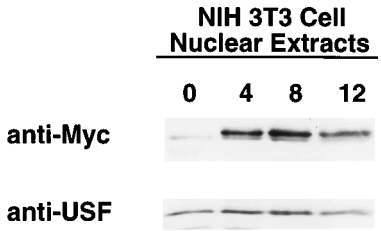

FIG. 5. Western analysis of basic helix-loop-helix proteins in serum-starved and -stimulated NIH 3T3 cells. Nuclear extracts were prepared from NIH 3T3 cells which had been serum starved (0) and stimulated for 4, 8, or 12 h, resolved by SDS-PAGE, and subjected to Western analysis. The same immunoblot was probed for c-Myc and USF. Bacterially expressed human c-Myc, Max, and USF were electrophoresed on the same gel for controls and were specifically detected by anti-c-Myc, anti-Max, and anti-USF antisera, respectively (data not shown).

Inhibitory mutants of c-Myc selectively suppresses *cad* transcription during S phase. To begin to dissect the functional contribution of the various E-box-binding factors to *cad* transcription in vivo, serum starvation and stimulation experiments were performed in the presence of inhibitory c-Myc proteins. Expression of the mutants MycΔBr and MycΔTAD has been shown to interfere with the function of Max-associated complexes in cultured cells by the following model (Fig. 6A). The mutants possess intact dimerization domains, allowing free association with cellular Max. However, since MycΔBr lacks the basic DNA-binding domain of c-Myc (10) and since MycΔTAD lacks most of the transactivation domain (32, 52), the resulting MycΔBr:Max and MycΔTAD:Max heterodimers are transcriptionally nonfunctional (1, 37). On the basis of several lines of biochemical evidence, continuous high-level expression of MycΔBr or MycΔTAD is believed to sequester limiting pools of cellular Max away from its endogenous partner proteins, thereby blocking their normal function. These and other similarly mutated c-Myc proteins have been shown to interfere (18 to 90%) in a *trans*-dominant fashion with exogenously added c-Myc in *myc*/*ras* cotransformation assays (14, 25, 52). Both also interfere with CACGTG-driven reporter activity in proliferating cells (1, 37). Moreover, since c-Myc does not associate with USF in vitro (10), it is believed that MycΔBr and MycΔTAD do not sequester USF or other E-box-binding factors that act independently of Max. Therefore, if G_1/S-phase transactivation of the *cad* promoter requires a Max-associated factor, both MycΔBr and MycΔTAD should inhibit *cad* transcription with the same efficiency. However, since MycΔTAD:Max complexes can bind DNA, the MycΔTAD mutant could also efficiently occlude DNA-binding sites recognized by other E-box factors. If a non-Max-associated factor is primarily responsive for *cad* transcription, MycΔTAD will be a more effective inhibitor than MycΔBr. Similar experiments with USF dominant-negative proteins cannot be performed, since existing mutant proteins are known to interfere with E-box proteins that are not normal partners of the wild-type USF.

To determine if MycΔBr and MycΔTAD could inhibit *cad* transcription during the growth cycle, the −81/+83 *cad* reporter was transfected into NIH 3T3 cells with plasmids expressing either no protein, MycΔBr, or MycΔTAD. On the basis of mutation of the E box in the −81/+83 *cad* promoter background, these inhibitory proteins would reduce G_1/S-phase activation to approximately 30% of the normal value if they were fully effective, provided, of course, that Max complexes were the primary contributors to growth-dependent transactivation. Figure 6B (right panel) represents the effect of the two mutant c-Myc expression plasmids on the induction of *cad* and another G_1/S-phase-activated promoter, *dhfr*, during S phase. *dhfr* served as a negative control, since E2F has been shown to be the key regulator of *dhfr* transcription throughout the growth cycle (62). As expected, the level of *dhfr* induction was not significantly affected by coexpression of MycΔBr or MycΔTAD, indicating that expression of the mutants did not block cells from entering S phase. In contrast, the level of *cad* induction was reduced to 40 to 46% of the S-phase value observed in cells expressing the vector control plasmid. This magnitude of suppression by MycΔBr and MycΔTAD was similar to that seen by others in experiments with antisense c-Myc (54). Furthermore, since both MycΔBr and MycΔTAD inhibited *cad* transcription to roughly the same degree, these data suggest that sequestration of Max, rather than binding-site occlusion, was the primary means of inhibition. The remaining fivefold induction in *cad* promoter activity was comparable to the fourfold induction of the E-box-mutated *cad* construct, cadEboxmt, in standard transfection experiments (Fig. 6B, left panel). This comparison suggests that coexpression of c-Myc inhibitory mutants eliminated most of the G_1/S-phase induction provided by the E-box element. Another E-box-binding factor(s) may have provided the remaining induction at the G_1/S-phase boundary, either as a normal regulator or as an opportunistic event in the absence of functional Max-associated complexes. Alternatively, expression of MycΔBr or MycΔTAD under these conditions may not have completely blocked the function of relevant Max complexes. These data suggest, therefore, that the factor(s) which regulates *cad* transcription in response to growth signals acts through a Max-associated complex that requires an E-box motif. Since the only Max partner known to transactivate transcription is the oncoprotein c-Myc, these data provide indirect evidence that c-Myc is involved in G_1/S-phase activation of *cad*, a nucleotide biosynthetic gene. Of course, we cannot exclude the possibility that the Myc mutant proteins have unpredicted effects on other E-box proteins. However, further evidence that supports a role for c-Myc as a potential upstream activator of *cad* comes from cotransfection assays in which the −81/+83 *cad* promoter construct was specifically activated threefold in the presence of plasmids expressing wild-type c-Myc versus no protein (data not shown). While this level of induction is modest, it is comparable to what has been reported for other c-Myc-activated genes (18, 59).

DISCUSSION

In the course of studying the activation of the *cad* gene at the G_1/S-phase boundary, we have demonstrated that the extended E-box sequence, CCACGTGG, which is located at +65 in the *cad* promoter, is a growth-dependent response element. Nuclear proteins bind the *cad* E box in a growth cycle-dependent manner in NIH 3T3 cells, and mutation or deletion of the E box results in a dramatically less inducible promoter throughout the G_1 and S phases. Introducing the E box into various unresponsive promoters conferred growth-dependent transcriptional regulation, indicating that this element is both necessary and sufficient for G_1/S-phase activation. In contrast, the E2F-like sequence which lies just 3′ to the E box does not substantially contribute to *cad* transcription at the G_1/S-phase boundary. This finding is consistent with earlier evidence that the *cad* promoter does not compete for E2F binding to the *dhfr* promoter (12) and that E2F does not transactivate *cad* in cotransfection assays (43). An interesting feature of the growth-responsive E box in the hamster *cad* promoter is that it lies in a transcribed but untranslated region of the gene. We

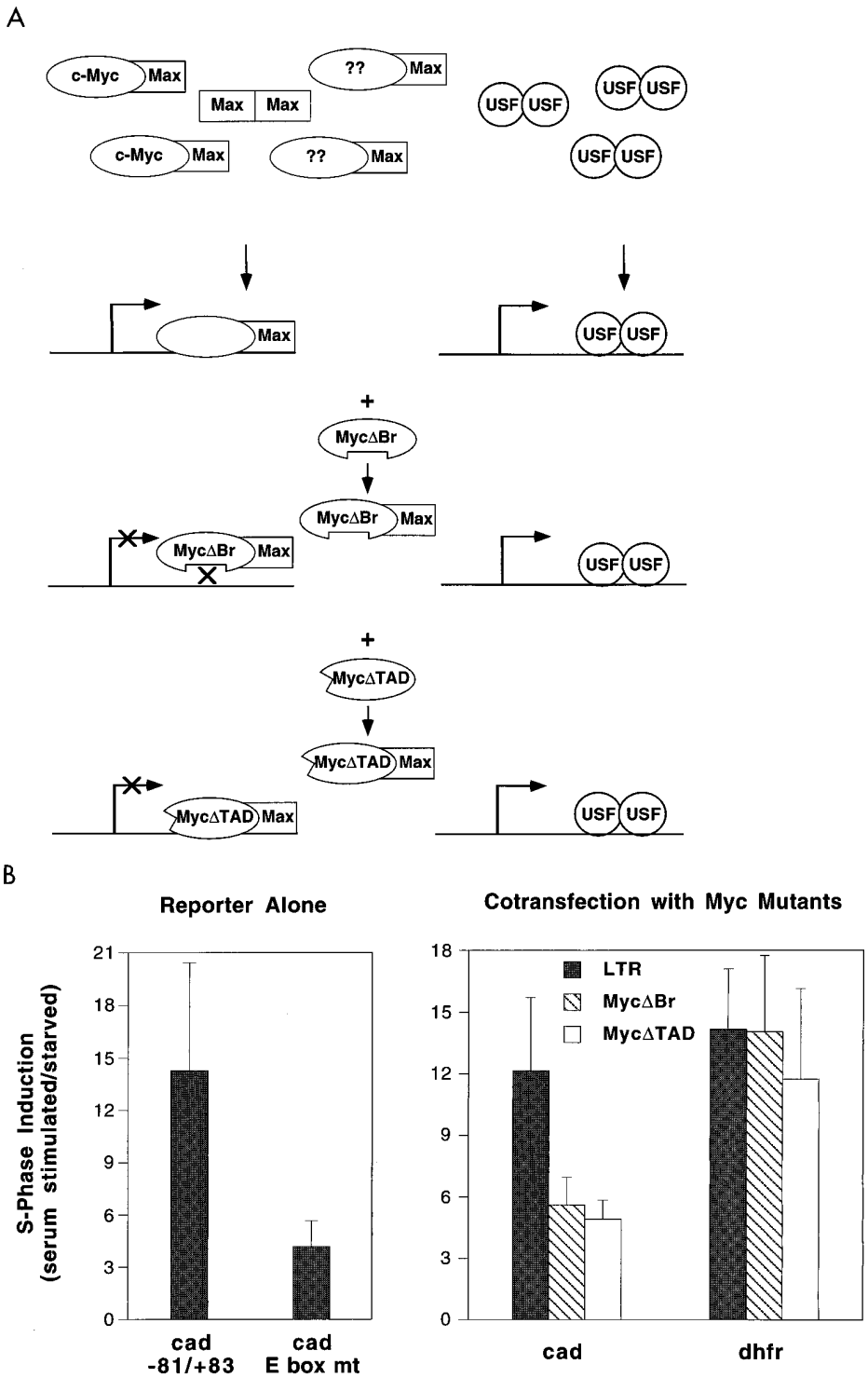

FIG. 6. Effect of expression of inhibitory c-Myc mutants on *cad* transcription during the S phase of the growth cycle. (A) Hypothetical schematic. Depicted (top) are various E-box-binding complexes present in mammalian cells, including USF, c-Myc:Max, Max homodimers, and a hypothetical (?) transactivation-competent Max-associated complex. G_1/S-phase transcription from the *cad* promoter may be regulated by a Max-associated complex (left) or USF (right). Expression of inhibitory c-Myc mutant proteins is proposed to specifically interfere with transactivation by Max-associated complexes but not transactivation by USF or other E-box-binding factors that act independently of Max. MycΔBr is proposed to sequester cellular Max into complexes which cannot bind DNA and therefore cannot transactivate. MycΔTAD is proposed to sequester Max into DNA-binding competent complexes which fail to transactivate and therefore occlude the E box. (B) Transcriptional activity in S phase. The left panel shows S-phase induction of the wild-type and E-box-mutated *cad* promoter. Data represent the peak promoter activity at 16 h reported in the time course graphs in Fig. 1. In the right panel, NIH 3T3 cells were transiently transfected with the expression plasmids LTR, LTR-MycΔBr (MycΔBr), or LTR-MycΔTAD (MycΔTAD) and either the cad−81/+83 reporter or the *dhfr* reporter pWTluc. Cells were harvested in duplicate at 0, 12, 14, and 16 h following serum stimulation. Luciferase activity was measured at each time point and averaged between duplicate samples. S-phase induction was determined by dividing the maximum promoter activity in serum-stimulated cells by the level in serum-starved cells. The maximum induction obtained in four to eight independent experiments was averaged and is represented here. Error bars indicate the standard error between experiments. Basal promoter activity was comparable between promoter constructs and was at least 10- to 150-fold above the luciferase activity measured in mock-transfected cells.

found that when the +55/+75 *cad* element was located downstream of +1, greater S-phase activity was consistently supported than when this element was located upstream. E-box sequences are located downstream of the transcription initiation site in several c-Myc-activated genes, such as *ECA39*, *p53*, and murine *odc*, suggesting that perhaps this location may confer an as yet unexplained selectivity to the site.

Since several cellular factors can affect transcription through the CCACGTGG motif, it is important to determine which E box-binding protein(s) modulates *cad* transcription. We showed that the binding activity of USF and Max and the protein levels of c-Myc changed in a growth cycle-dependent manner that closely correlates with *cad* promoter activity. Of these E-box-binding proteins, c-Myc is the only one shown previously to affect growth cycle progression, so it was a logical candidate for the regulator of *cad* transcription. Consistent with this hypothesis, c-Myc could activate transcription from the *cad* promoter, and *cad* transcription in S-phase was selectively inhibited by two dominant-negative c-Myc mutants that have been shown by others to decrease activity of c-Myc:Max heterodimers in both transcription and cellular transformation assays. This result suggests that the factor(s) which regulates the E-box-mediated increase in *cad* promoter activity at the G_1/S-phase transition may act in the same pathway as c-Myc and/or Max but does not prove that these factors play a direct role. Although suppression of *cad* transcription by inhibitory c-Myc proteins was significant, it was not complete, suggesting that other E-box-binding factors may collectively contribute to *cad* promoter activity through the growth cycle. We do not believe that the other Max-associated factors, Mad and Mxi1, play a role in G_1/S-phase activation of *cad*, because they have been implicated as differentiation-specific transcriptional repressors. It will be interesting, however, to determine in future studies whether Mad:Max and/or Mxi1:Max contributes to down-regulation of *cad* gene expression upon differentiation.

In summary, our studies involving promoter mutagenesis and DNA-binding assays indicate that E-box proteins are key regulators of *cad* transcription at the G_1/S-phase transition. The use of inhibitory mutants suggests that a Max-associated complex contributes to growth-dependent regulation of *cad*. On the basis of well-established data about the various Max partner proteins to date, our observations point to c-Myc:Max heterodimers as potentially significant regulators of *cad* transcription during the transition from quiescence to a proliferative state. More direct studies are needed in vivo to precisely determine to what extent the other E-box-binding proteins, such as USF, contribute to G_1/S-phase activation. Finally, the fact that c-Myc and E2F1 can both transform cells in culture and are both required for G_1/S-phase progression raises the question whether c-Myc and E2F regulate checkpoints in the cell cycle via a linear pathway. Our finding that dominant-negative mutants of c-Myc do not interfere with activation of an E2F-regulated gene, *dhfr*, provides one piece of evidence that c-Myc is not an obligate, upstream component of the E2F signal transduction pathway leading to S phase.

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REFERENCES

1. **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.
2. **Ayer, D. E., and R. N. Eisenman.** 1993. A switch from Myc:Max to Mad:Max hetercomplexes accompanies monocyte/macrophage differentiation. Genes Dev. **7:**2110–2119.
3. **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.
4. **Bandara, L. R., V. M. Buck, M. Zamanian, L. H. Johnston, and N. B. La Thangue.** 1993. Functional synergy between DP-1 and E2F-1 in the cell cycle-regulating transcription factor DRTF1/E2F. EMBO J. **12:**4317–4324.
5. **Beckmann, H., L. Su, and T. Kadesch.** 1990. TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer μE3 motif. Genes Dev. **4:**167–179.
6. **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.
7. **Bendall, A. J., and P. L. Molloy.** 1994. Base preferences for DNA binding by the bHLH-Zip protein USF: effects of $MgCl_2$ on specificity and comparison with binding of Myc family members. Nucleic Acids Res. **22:**2801–2810.
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:**2513–2523.
9. **Berberich, S., N. Hyde-DeRuyscher, 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. **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.
11. **Blackwood, E. M., B. Lüscher, and R. N. Eisenman.** 1992. Myc and Max associate in vivo. Genes Dev. **6:**71–80.
12. **Buermeyer, A. B., N. E. Thompson, L. A. Strasheim, R. R. Burgess, and P. J. Farnham.** 1992. The HIP1 initiator element plays a role in determining the in vitro requirement of the dihydrofolate reductase gene promoter for the C-terminal domain of RNA polymerase II. Mol. Cell. Biol. **12:**2250–2259.
13. **Carr, C. S., and P. A. Sharp.** 1990. A helix-loop-helix protein related to the immunoglobulin E box-binding proteins. Mol. Cell. Biol. **10:**4384–4388.
14. **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.
15. **Dean, M., R. A. Levine, W. Ran, M. S. Kindy, G. E. Sonenshein, and J. Campisi.** 1986. Regulation of c-*myc* transcription and mRNA abundance by serum growth factors and cell contact. J. Biol. Chem. **261:**9161–9166.
16. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
17. **Dou, Q.-P., S. Zhao, A. H. Levin, J. Wang, K. Helin, and A. B. Pardee.** 1994. G_1/S-regulated E2F-containing protein complexes bind to the mouse thymidine kinase gene promoter. J. Biol. Chem. **269:**1306–1313.
18. **Eilers, M., S. Schirm, and J. M. Bishop.** 1991. The MYC protein activates transcription of the α-prothymosin gene. EMBO J. **10:**133–141.
19. **Eisenman, R. N., C. Y. Tachibana, H. D. Abrams, and S. R. Hann.** 1985. v-*myc* and c-*myc*-encoded proteins are associated with the nuclear matrix. Mol. Cell. Biol. **5:**114–126.
20. **Evan, G. I., and T. D. Littlewood.** 1993. The role of c-*myc* in cell growth. Curr. Opin. Genet. Dev. **3:**44–49.
21. **Evans, D. R.** 1986. CAD, a chimeric protein that initiates de novo pyrimidine biosynthesis in higher eucaryotes, p. 283–331. *In* D. G. Hardie and J. R. Coggins (ed.), Multidomain proteins—structure and evolution. Elsevier Biomedical Press, Amsterdam.
22. **Farnham, P. J., and R. Kollmar.** 1990. Characterization of the 5′ end of the growth-regulated syrian hamster CAD gene. Cell Growth Differ. **1:**179–189.
23. **Farnham, P. J., J. E. Slansky, and R. Kollmar.** 1993. The role of E2F in the mammalian cell cycle. Biochim. Biophys. Acta **1155:**125–131.
24. **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.
25. **Halazonetis, T. D., and A. N. Kandil.** 1991. Determination of the c-MYC DNA-binding site. Proc. Natl. Acad. Sci. USA **88:**6162–6166.
26. **Heikkila, R., G. Schwab, E. Wickstrom, S. L. Loke, D. H. Pluznik, R. Watt, and L. M. Neckers.** 1987. A c-*myc* antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G_0 to G_1. Nature (London) **328:**445–449.

27. **Helin, K., C.-L. Wu, A. R. Fattaey, J. A. Lees, B. D. Dynlacht, C. Ngwu, and E. Harlow.** 1993. Heterodimerization of the transcription factor E2F-1 and DP1 leads to cooperative trans-activation. Genes Dev. **7:**1850–1861.
28. **Hsiao, K.-M., S. L. McMahon, and P. J. Farnham.** 1994. Multiple DNA elements are required for the growth regulation of the mouse *E2F1* promoter. Genes Dev. **8:**1526–1537.
29. **Hu, Y., B. Lüscher, A. Admon, N. Mermod, and R. Tjian.** 1990. Transcription factor AP-4 contains multiple dimerization domains that regulate dimer specificity. Genes Dev. **4:**1741–1752.
30. **Ivey-Hoyle, M., R. Conroy, H. E. Huber, P. J. Goodhart, A. Oliff, and D. C. Heimbrook.** 1993. Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. Mol. Cell. Biol. **13:**7802–7812.
31. **Johnson, D. G., J. K. Schwarz, W. D. Cress, and J. R. Nevins.** 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature (London) **365:**349–352.
32. **Kato, G. J., J. Barrett, M. Villa-Garcia, and C. V. Dang.** 1990. An amino-terminal domain of c-Myc required for transformation activates transcription. Mol. Cell. Biol. **10:**5914–5920.
33. **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.
34. **Kollmar, R., M. J. Lindstrom, and P. J. Farnham.** 1992. Heat sensitivity and Sp1 activation of complex formation at the Syrian hamster carbamoyl-phosphate synthase (glutamine-hydrolyzing)/aspartate carbamoyltransferase/dihydroorotase promoter *in vitro*. J. Biol. Chem. **267:**385–391.
35. **Kollmar, R., K. A. Sukow, S. K. Sponagle, and P. J. Farnham.** 1994. Start site selection at the TATA-less carbamoyl-phosphate synthase (glutamine-hydrolyzing)/aspartate carbamoyltransferase/dihydroorotase promoter. J. Biol. Chem. **269:**2252–2257.
36. **Krek, W., D. M. Livingston, and S. Shirodkar.** 1993. Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. Science **262:**1557–1560.
37. **Kretzner, L., E. M. Blackwood, and R. N. Eisenman.** 1992. Myc and Max proteins possess distinct transcriptional activities. Nature (London) **359:**426–429.
38. **Lam, E. W.-F., J. D. H. Morris, R. Davies, T. Crook, R. J. Watson, and K. H. Vousden.** 1994. HPV16 E7 oncoprotein deregulates B-*myb* expression: correlation with targeting of p107/E2F complexes. EMBO J. **13:**871–878.
39. **Lam, E. W.-F., and R. J. Watson.** 1993. An E2F-binding site mediates cell-cycle regulated repression of mouse B-*myb* transcription. EMBO J. **12:**2705–2713.
40. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304:**596–602.
41. **Letovsky, J., and W. S. Dynan.** 1989. Measurement of the binding of transcription factor Sp1 to a single GC box recognition sequence. Nucleic Acids Res. **17:**2639–2653.
42. **Li, L., G. S. Naeve, and A. S. Lee.** 1993. Temporal regulation of cyclin A-p107 and p33cdk2 complexes binding to a human thymidine kinase promoter element important for G1-S phase transcriptional regulation. Proc. Natl. Acad. Sci. USA **90:**3554–3558.
43. **Li, Y., J. E. Slansky, D. J. Myers, N. R. Drinkwater, W. G. Kaelin, and P. J. Farnham.** 1994. Cloning, chromosomal location, and characterization of mouse *E2F1*. Mol. Cell. Biol. **14:**1861–1869.
44. **Liao, W. S. L., R. Heller, P. Green, and G. R. Stark.** 1986. Regulation of carbamoyl phosphate synthetase-aspartate transcarbamoylase-dihydroorotase gene expression in growing and arrested cells. J. Biol. Chem. **261:**15577–15581.
45. **Lim, K., and C.-B. Chae.** 1989. A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for beta-galactosidase. BioTechniques **7:**576–579.
46. **Lüscher, B., and R. N. Eisenman.** 1990. New light on Myc and Myb. I. Myc. Genes Dev. **4:**2025–2035.
47. **Makino, R., K. Hayashi, and T. Sugimura.** 1984. c-*myc* transcript is induced in rat liver at a very early stage of regeneration or by cycloheximide treatment. Nature (London) **310:**697–698.
48. **Marcu, K. B., S. A. Bossone, and A. J. Patel.** 1992. *myc* function and regulation. Annu. Rev. Biochem. **61:**809–860.
49. **Means, A. L., J. E. Slansky, S. L. McMahon, M. W. Knuth, and P. J. Farnham.** 1992. The HIP1-binding site is required for growth regulation of the dihydrofolate reductase gene promoter. Mol. Cell. Biol. **12:**1054–1063.

49a. **Miltenberger, R. J.** Unpublished data.

50. **Miltenberger, R. J., J. Cortner, and P. J. Farnham.** 1993. An inhibitory Raf-1 mutant suppresses expression of a subset of v-*raf*-activated genes. J. Biol. Chem. **268:**15674–15680.
51. **Mudryj, M., S. W. Hiebert, and J. R. Nevins.** 1990. A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. EMBO J. **9:**2179–2184.
52. **Mukherjee, B., S. D. Morgenbesser, and R. A. DePinho.** 1992. Myc family oncoproteins function through a common pathway to transform normal cells in culture: cross-interference by Max and *trans*-acting dominant mutants. Genes Dev. **6:**1480–1492.
53. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
54. **Peña, A., C. D. Reddy, S. Wu, N. J. Hickok, E. P. Reddy, G. Yumet, D. R. Soprano, and K. J. Soprano.** 1993. Regulation of human ornithine decarboxylase expression by the c-Myc:c-Max protein complex. J. Biol. Chem. **268:**27277–27285.
55. **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.
56. **Prochownik, E. V., J. Kukowska, and C. Rodgers.** 1988. c-*myc* antisense transcripts accelerate differentiation and inhibit G_1 progression in murine erythroleukemia cells. Mol. Cell. Biol. **8:**3683–3695.
57. **Rabbitts, P. H., J. V. Watson, A. Lamond, A. Forster, M. A. Stinson, G. Evan, W. Fischer, E. Atherton, R. Sheppard, and T. H. Rabbitts.** 1985. Metabolism of c-*myc* gene products: c-*myc* mRNA and protein expression in the cell cycle. EMBO J. **4:**2009–2015.
58. **Rao, G. N., and R. L. Church.** 1988. Regulation of CAD gene expression in mouse fibroblasts during the transition from the resting to the growing state. Exp. Cell Res. **178:**449–456.
59. **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.
60. **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.
61. **Singh, P., S. H. Wong, and W. Hong.** 1994. Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. EMBO J. **13:**3329–3338.
62. **Slansky, J. E., Y. Li, W. G. Kaelin, and P. J. Farnham.** 1993. A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. Mol. Cell. Biol. **13:**1610–1618. (Author's correction, **13:**7201.)
63. **Solomon, D. L. C., B. Amati, and H. Land.** 1993. Distinct DNA binding preferences for the c-Myc/Max and Max/Max dimers. Nucleic Acids Res. **21:**5372–5376.
64. **Thompson, C. B., P. B. Challoner, P. E. Neiman, and M. Groudine.** 1985. Levels of c-*myc* oncogene mRNA are invariant throughout the cell cycle. Nature (London) **314:**363–366.
65. **Waters, C. M., T. D. Littlewood, D. C. Hancock, J. P. Moore, and G. I. Evan.** 1991. c-*myc* protein expression in untransformed fibroblasts. Oncogene **6:**797–805.
66. **Watt, R., L. W. Stanton, K. B. Marcu, R. C. Gallo, C. M. Croce, and G. Rovera.** 1983. Nucleotide sequence of cloned cDNA of human c-*myc* oncogene. Nature (London) **303:**725–728.
67. **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.