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Myc versus USF: Discrimination at the *cad* Gene Is Determined by Core Promoter Elements

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Carbamoyl-phosphate synthase/aspartate carbamoyltransferase/dihydroorotase, which is encoded by the *cad* gene, is required for the first three rate-limiting steps of de novo pyrimidine biosynthesis. It has been previously demonstrated that *cad* transcription increases at the G_1/S-phase boundary, as quiescent cells reenter the proliferative cell cycle. The growth-responsive element has been mapped to an E box at +65 in the hamster *cad* promoter. Using an in vivo UV cross-linking and immunoprecipitation assay, we show that Myc, Max, and upstream stimulatory factor (USF) bind to the chromosomal *cad* promoter. To determine whether binding of Myc-Max or USF is critical for *cad* growth regulation, we analyzed promoter constructs which contain mutations in the nucleotides flanking the E box. We demonstrate that altering nucleotides which flank the *cad* E box to sequences which decrease Myc-Max binding in vitro correlates with a loss of *cad* G_1/S-phase transcriptional activation. This result supports the conclusion that binding of Myc-Max, but not USF, is essential for *cad* regulation. Our investigations demonstrate that the endogenous *cad* E box can be bound by more than one transcription factor, but growth-induced *cad* expression is achieved only by Myc.

The product of the c-*myc* proto-oncogene is a critical regulator of cellular proliferation (30). In accordance with this role, overexpression of c-*myc* contributes to the transformation of primary fibroblasts in culture (34) as well as to the development of neoplasia in a wide variety of tissues (for a review, see reference 39). In response to growth signals, levels of c-*myc* mRNA and protein are increased rapidly, peak in G_1 phase, and return to lower invariant levels throughout the rest of the cell cycle (16, 32). The function of Myc in cellular transformation and cell cycle progression is dependent on dimerization with its cellular partner Max (1, 2). One mechanism by which Myc-Max heterodimers are thought to control cellular proliferation is through their ability to transcriptionally activate downstream target genes. Potential genes which have been suggested to be candidates for regulation by Myc-Max include *odc* (8, 51, 52), α-*prothymosin* (19, 25), *p53* (47), *ECA39* (10), *eIF4E* (31), *cdc25* (24), *MrDb* (27), and *cad* (43). Mutational analysis has shown that all of these genes contain Myc-Max binding sites which contribute to promoter activity. However, based on the expression pattern of c-*myc*, an additional requirement for Myc target genes should include growth-regulated transcriptional activity. Growth-regulated promoter activity has been demonstrated for some, but not all, of the putative Myc target genes.

In our laboratory, we study the hamster *cad* gene, which encodes a trifunctional enzyme (carbamoyl-phosphate synthetase/aspartate carbamoyltransferase/dihydroorotase [CAD]) required for the first three rate-limiting steps of de novo pyrimidine biosynthesis. In the cell, CAD functions to increase the pools of free nucleotides needed for the synthesis of new DNA. Both CAD enzymatic activity and the expression of *cad* mRNA are dependent on the proliferative state of the cell. For example, in tumor cells, an increased growth rate has been correlated with a rise in CAD activity (4). It has also been shown that levels of endogenous *cad* mRNA increase approximately 10-fold in late G_1 when quiescent fibroblasts are stimulated to begin proliferating (13, 46). Conversely, a sharp decrease in the amount of *cad* mRNA is detected as HL60 cells become terminally differentiated (44). The regulation of *cad* gene expression occurs primarily at the level of transcription (36, 45). Using transient transfection assays, we previously mapped the growth-responsive element within the hamster *cad* promoter to sequences centered at +65, which contain a Myc-Max binding site. We have also shown that increased expression from the *cad* promoter at the G_1/S-phase boundary is suppressed by dominant negative Myc proteins, suggesting that Myc is directly involved in regulating *cad* transcription (43). Thus, *cad* meets the requirements for a potential Myc target gene; the promoter is growth regulated, and this regulation is mediated by a Myc-Max binding site.

Studies of potential Myc target genes are complicated by the fact that Myc-Max heterodimers recognize the hexanucleotide E-box element CANNTG (where N is A, C, G, or T), which is also the binding motif for several different cellular factors. These proteins include the constitutively present transcription factors TFE3 (6), TFEB (17), and upstream stimulatory factor (USF) (28), as well as proteins involved in the Myc network, which include the heterodimeric Myc-Max, Mad-Max, and Mxi-Max complexes (5, 12, 55). Although in vitro assays suggest that each of these factors can recognize the same core sequence of CACGTG, it is possible that in vivo, certain of these proteins achieve specific binding to a subset of E boxes. To further investigate which E-box-binding factor regulates *cad* expression, we have performed in vivo UV cross-linking to examine binding specificity of the chromosomal *cad* E box. Here, we demonstrate that Myc, Max, and USF can all bind to the *cad* E box in vivo. Therefore, to determine which protein is responsible for the growth regulation of the *cad* promoter, it was necessary to create *cad* promoter constructs that were altered in the ability to bind each of these transcription factors. Since it has been previously demonstrated that discrimination between USF and Myc-Max binding in vitro can be achieved through the sequences which flank the core consensus E box (9, 23), we created *cad* promoter constructs which contain a series of mutations in the nucleotides immediately flanking the

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cad E box. Here, we show a correlation between E-box elements which favor Myc-Max binding in vitro and the G_1/S-phase regulation of *cad* expression in vivo. Taking into account both the in vitro and in vivo data, we suggest that although the wild-type *cad* E box may be bound by either USF or Myc-Max, growth-regulated expression is achieved only when the site is occupied by Myc-Max.

MATERIALS AND METHODS

Plasmids. Construction of the *cad* reporter plasmids cad −81/+83 and cad −81/+26, which contain *cad* promoter fragments cloned upstream of the luciferase cDNA in the pGL2Basic vector, has been described previously (33). Plasmids −81/+26[wt], −81/+26[mt1], −81/+26[mt2], −81/+26[mt3], and −81/+26[mt4] were constructed by inserting a double-stranded oligonucleotide corresponding to the endogenous hamster *cad* promoter sequence from +55 to +75 or mutations thereof and containing *Hin*dIII ends into the *Hin*dIII site within the pGL2Basic polylinker of the *cad* −81/+26 reporter construct. The −81/+26mtI[wt] construct was created by cloning the wt (wild-type) oligonucleotide into the *Hin*dIII site of linker-scanner mutant pCM-3][+3GL (33). Oligonucleotide sequences are as follows (boldface indicates a change from the wild-type *cad* sequence [the E box is underlined]): [wt], 5′AGCGAGCCACGTGGACCAACT; mt1, 5′AGCGAA**C**CACGTG**G**TCCAACT; mt2, 5′AGCGAG**T**CACGTG**A**ACCAACT; mt3, 5′AGCGA**AT**CACGTG**AC**CCAACT; mt4, 5′AGCGA**AT**CACATG**AC**CCAACT; and mtE, 5′AGCGAGC**CTGCAG**GACCAACT.

Cell culture and transfections. NIH 3T3 cell cultures were maintained at 37°C and 5% CO_2 in Dulbecco modified Eagle medium with high glucose (GIBCO) supplemented with 5% bovine calf serum (HyClone), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. One day prior to transfection, 60-mm-diameter dishes were seeded with 1.5×10^5 cells in maintenance medium. Cells were transiently transfected with 1 μg of *cad* reporter plasmid and 14 μg of sonicated salmon sperm carrier DNA, using a standard calcium phosphate protocol (41). After the cells were incubated at 37°C for 5 h in medium containing the transfection DNA, cells were shocked with 15% glycerol in HEPES buffer (42), rinsed with Dulbecco modified Eagle medium, and maintained in low-serum (0.5% bovine calf serum) medium for 48 h. Following serum starvation, parallel cultures were stimulated to grow by replacing low-serum medium with high-serum (10% serum) medium. Cells were harvested for luciferase activity (42) either prior to serum stimulation or at the indicated times following stimulation. Progression of the cells through the growth cycle was monitored by flow cytometric analysis of parallel dishes of cells harvested and stained with propidium iodide (50). All transfections were performed in duplicate, at least three times per construct, and with multiple DNA preparations.

Syrian baby hamster kidney B5-4 cells were maintained as suspension cultures at 37°C and 5% CO_2 in Joklik's suspension-modified Eagle medium (S-MEM) supplemented with 5% fetal bovine serum (GIBCO), 2 mM glutamine (Sigma), 1× nonessential amino acids (Sigma), 100 U of penicillin per ml, and 100 μg of streptomycin (GIBCO) per ml. These cells were previously selected in *N*-(phosphonacetyl)-L-aspartate (PALA) to contain 50 copies of the *cad* gene (36). Since the amplified copies of *cad* are stable, B5-4 cultures were routinely maintained in the absence of PALA. Cell density was maintained below 4×10^5 cells/ml.

In vitro translation and electromobility shift assays. Transcription plasmids pBS 0/1 Myc and pVZ1 p21 Max (gifts of R. N. Eisenman) were linearized with *Dra*I and *Not*I, respectively. Transcripts were made by T3 polymerase. Rabbit reticulocyte lysates (Promega) were programmed with 29 ng of Myc RNA or 114 ng of Max RNA per μl of reticulocyte lysate as instructed by the manufacturer. [^{35}S]methionine was included in parallel reactions, and the resulting products were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to monitor translation efficiency. Equal amounts of Max programmed lysate and Myc programmed lysate were incubated together at 30°C for 45 min to form Myc-Max heterodimers. All lysates were stored at −70°C.

Electromobility shift assays were performed as previously described (43), with the following modifications. Binding reaction mixtures 19-μl total volume contained 11 μl of pH 7.0 binding buffer, 2 μg of sonicated salmon sperm DNA, and 1 μl of reticulocyte lysate as indicated: unprogrammed reticulocyte lysate, Myc and Max lysates which were preincubated together for 45 min, or 2 μg of HeLa nuclear extract (41) as a source of USF protein plus Myc and Max lysates which were preincubated together for 45 min. Where specified, binding reaction mixtures were incubated for 20 min with either a 50-fold molar excess of unlabeled probe oligonucleotide as a competitor or 2 μg of polyclonal antibody prior to the addition of double-stranded oligonucleotide probes, which were end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (49). Upon addition of the probe, binding reaction mixtures were incubated for an additional 20 min at room temperature and then resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide, 29:1) for 2 h. The gel was preelectrophoresed for 60 min. Gels were dried, and protein-DNA interactions were visualized by autoradiography. The gel and running buffer contained 22.5 mM Tris-morpholinepropanesulfonic acid (pH 7.0) and 0.5 mM EDTA. Antibodies (anti-Max sc-765X, anti-c-Myc sc-764X, and anti-USF sc-229X) were purchased from Santa Cruz Biotechnology.

Western immunoblot analysis. B5-4 cells (4×10^6; log-phase cultures) were swollen in 600 μl of hypotonic reticulocyte standard buffer (RSB) (20) supplemented with 0.5 mM dithiothreitol, 100 μg of phenylmethysulfonyl fluoride (PMSF) per ml, 1 μg of orthovanadate per ml, and 10 μg of leupeptin per ml. Nonidet P-40 (NP-40; 0.5%, vol/vol) was added to swollen cells, and nuclei were released by Dounce homogenization. Nuclei were pelleted by centrifugation at $4{,}000 \times g$ for 5 min. Pellets were washed once in RSB and then resuspended in 100 μl RSB supplemented with 0.5% deoxycholate, 1.0% octyl-β-glucoside, 0.5 mM dithiothreitol, 100 μg of PMSF per ml, 1 μg of orthovanadate per ml, and 10 μg of leupeptin per ml. Lysed nuclei were passaged 10 times through a 22-gauge needle. One-third of the lysate (1.3×10^6 cells) was loaded per lane and resolved by SDS-PAGE under reducing conditions on a 13% polyacrylamide gel (29:1, acrylamide/bisacrylamide) by standard techniques (49). Proteins were transferred to nitrocellulose and probed separately with 2 μg of rabbit polyclonal antibody (Santa Cruz) (see below) overnight at 4°C. Blots were then probed with a 1:5,000 dilution of peroxidase-conjugated goat anti-rabbit secondary antibody (sc-2004; Santa Cruz). Proteins were detected by enhanced chemiluminescence (Kirkegaard & Perry).

UV cross-linking and isolation of chromatin. The UV cross-linking and immunoprecipitation protocol was performed as a modification of the procedure previously described by Walter et al. (54). Cultures of 3×10^8 B5-4 cells were collected by centrifugation (Beckman GS-6R) at 1,000 rpm for 5 min, washed in cold 1× phosphate-buffered saline (PBS), resuspended in 3 ml of 1× PBS on ice, and transferred to a 100-mm-diameter standard tissue culture dish. Cells were subjected to UV cross-linking by placing dishes 3 cm below five UV bulbs (254 nm) in a Stratalinker (model 1800; Stratagene) for 6 min on ice. Cells were collected by centrifugation at $2{,}000 \times g$ for 10 min. The resulting cell pellets were resuspended in nuclei lysis buffer (100 mM NaCl, 10 mM Tris [pH 8.0], 1 mM EDTA, 0.1% NP-40, 0.5 mM PMSF) in a B Dounce homogenizer. Sarkosyl was added to a final concentration of 2%, and the nuclear lysates were vortexed. Chromatin in nuclear lysates was sheared by two passages through a 22-gauge needle followed by four passages through a 26-gauge needle. Protein-cross-linked DNA was isolated from unbound protein and RNA by centrifugation through a 2.9-ml CsCl gradient (1.3 ml of 1.33-g/ml, 0.9 ml of 0.8-g/ml, and 0.7 ml of 0.44-g/ml CsCl in 0.5% Sarkosyl–1 mM EDTA) in an SW60 rotor for 20 h at 30,000 rpm and 20°C. Peak DNA-containing fractions, identified by agarose gel electrophoresis and ethidium bromide (EtBr) staining, were pooled and dialyzed with Spectra/Por no. 2 tubing against 2 mM EDTA–50 mM Tris (pH 8.0). Chromatin was frozen at −70°C in aliquots corresponding to 5×10^7 cells.

Immunoprecipitation of chromatin. Aliquoted chromatin was subjected to *Bam*HI restriction digestion at 37°C. Digestion reaction mixtures of 1.7 ml containing 200 U of *Bam*HI, 150 mM NaCl, 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, and 0.01% Triton X-100 were incubated for 8 h. An additional 100 U of *Bam*HI was added, and digestion was continued for another 12 h. A third 100-U aliquot of *Bam*HI plus 60 μg of RNase A (Boehringer Mannheim) was then added, and digestion continued for another 2 h. After verification that digestion was complete by electrophoresis on a 0.7% agarose gel, digestion reactions were stopped by the addition of a 1/25 volume of 0.5 M EDTA–Sarkosyl to a final concentration of 0.05% and Triton X-100 to a final concentration of 0.3%. Ten percent of the digest was reserved as a sample representing 10% of total input DNA and processed in the proteinase K step described below. Two micrograms of 8WG16 mouse monoclonal antibody (gift of the R. Burgess laboratory), 2 μg of affinity-purified rabbit polyclonal antibody (anti-Myc sc-788, anti-Max sc-765X, or anti-USF sc-229X; Santa Cruz), or 2 μg of affinity-purified rabbit anti-mouse immunoglobulin G (IgG)-IgA-IgM (heavy plus light chain) polyclonal antibody (Zymed 61-6400) was added to digested chromatin. The reaction mixtures were rotated at 4°C for 3 h. Four micrograms of rabbit anti-mouse polyclonal antibody was added to the 8WG16 reaction mixture, which was then incubated for an additional hour with rotation at 4°C. Chromatin was immunoprecipitated by incubation with prepared Staph A cells (see below) which were preincubated with sonicated HeLa chromatin (UV cross-linked and prepared as described above for B5-4 chromatin) for 3 h at 4°C to block nonspecific DNA-Staph A interactions. Blocked Staph A cells were washed twice in 200 μl of dialysis buffer (2 mM EDTA, 50 mM Tris [pH 8.0]) and resuspended in 100 μl of dialysis buffer. Twenty-five microliters of the blocked Staph A solution was added to each antibody reaction and rotated at room temperature for 15 min. To prepare Staph A cells, 1 g of lyophilized Staph A cells (Boehringer Mannheim) was washed twice in dialysis buffer plus 0.2% Sarkosyl and boiled for 30 min in 2 volumes of PBS–3% SDS–10% β-mercaptoethanol. After being washed twice in dialysis buffer plus 0.2% Sarkosyl, the Staph A cells were frozen in liquid N_2 as a 20% solution in dialysis buffer plus 0.2% Sarkosyl in 100-μl aliquots.

Immunoprecipitates were pelleted by microcentrifugation at 15,000 rpm for 2 min. Staph A pellets were washed twice in 1.4 ml of dialysis buffer and four times in 1.4 ml of immunoprecipitation buffer (11 mM Tris [pH 8.0], 500 mM LiCl, 1% NP-40, 1% deoxycholic acid). Between washes, cells were pelleted at 15,000 rpm for 2 min in a microcentrifuge. Immunoprecipitates were eluted from the Staph A cells by three rounds of gentle vortexing of Staph A complexes for 10 min each in 100 μl of elution buffer (50 mM $NaHCO_3$, 1% SDS, 1.5 μg of sonicated salmon sperm DNA per ml) and microcentrifugation at 15,000 rpm for 2 min to collect the supernatant. To a total elution volume of 300 μl, 200 μl of proteinase K solution (0.3% SDS, 50 mM Tris [pH 7.5], 10 mM EDTA, 1 mg of proteinase

K per ml) was added, and samples were incubated at 60°C for 16 h. The 10% total DNA sample, reserved previously, was adjusted with H_2O to 300 μl and processed with proteinase K solution. DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate (NaOAc), pH 5.3, 20 μg of tRNA, and 2.5 volumes of ethanol (EtOH) and incubated at −20°C for 1 h and −70°C for 30 min, followed by microcentrifugation at 15,000 rpm for 15 min. DNA pellets were resuspended in 10 μl of 1× loading dye (10 mM sucrose, 0.16× TEA, 0.8 mM EDTA, 0.16 mg of bromophenol blue per ml) containing 60 ng of RNase A per μl for Southern blot analysis.

Southern blot analysis. Samples were electrophoresed in a 0.7% agarose gel (no EtBr), stained with 1.5 μg of EtBr per ml in 1× TEA for 10 min, destained in 1× TEA for 5 min, and photographed alongside a fluorescent ruler. The gel was denatured for 30 min in 1.5 M NaCl–0.5 M NaOH and neutralized for 30 min in 1.5 M NaCl–0.5 M Tris (pH 7.2)–1 mM EDTA (pH 8.0). Samples were transferred onto a Hybond-N membrane (Amersham) with 20× SSPE (3.6 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA) overnight, using standard capillary transfer. The blot was baked at 80°C for 30 min, UV cross-linked at 120,000 μJ, and incubated at 42°C overnight in prehybridization solution (50% formamide, 3.4× SSPE, 100 μg of sonicated salmon sperm DNA per ml, 50 μg of boiled sonicated salmon sperm DNA per ml, 5× Denhardt's solution, 10% dextran sulfate, 5% SDS, 1% Sarkosyl). The probe was labeled by random priming (Amersham 1601Z) 25 ng of DNA with [α-^{32}P]dCTP to a specific activity of approximately 3×10^5 cpm/ng. Labeled probe was added to the prehybridization solution (8×10^5 cpm/ml) and incubated at 42°C for 48 h. Blots were washed in 2× SSPE–0.1% SDS at room temperature for 30 min, 1× SSPE–0.1% SDS at 65°C for 15 min, and 0.5× SSPE–0.2% SDS at 65°C for 1 h. Blots were exposed to BioMax film (Kodak) for 24 to 48 h.

RESULTS

In vivo cross-linking identifies protein-DNA interactions at the chromosomal *cad* promoter. Previous studies have implicated an E box as the element which specifies G_1/S-phase regulation of the *cad* promoter. However, it remained unclear which cellular factor(s) binds the *cad* E box to confer regulation. To address this question, we used in vivo UV cross-linking to study protein-DNA interactions at the *cad* promoter. In vivo cross-linking provides a method to examine protein-DNA interactions as they occur under physiological conditions within the context of native chromatin structure. The UV cross-linking and immunoprecipitation protocol used in our experiments is outlined in Fig. 1A. Briefly, cell cultures are subjected to UV irradiation, causing covalent cross-links between DNA and protein which are in close contact. Cross-linked chromatin is digested with restriction enzymes that cut in the sequences flanking the binding site of interest, yielding a fragment of known size. DNA-protein complexes are immunoprecipitated by the addition of antibodies specific to DNA-cross-linked proteins. Immunoprecipitates from different antibodies are tested by Southern blotting for the presence of the DNA of interest. Ultimately, the ability of an antibody to immunoprecipitate a select DNA fragment suggests that the antigen protein is covalently attached to the fragment via its association with this DNA in vivo.

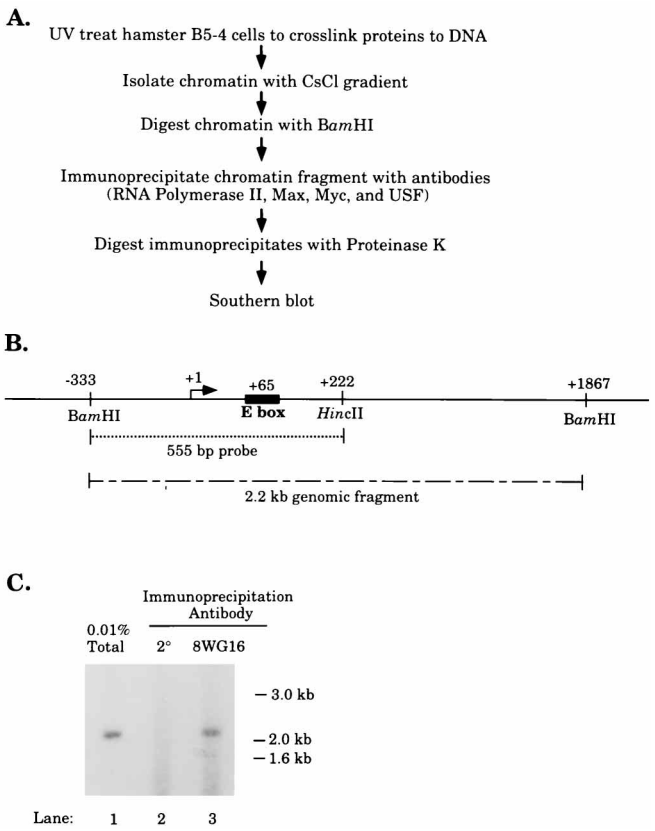

FIG. 1. Experimental approach for identifying protein-DNA interactions at the *cad* promoter in vivo. (A) General outline of the UV cross-linking immunoprecipitation technique used to identify DNA-bound proteins. This technique was adapted for mammalian cell culture from previous studies of *Drosophila* proteins by Walter et al. (54). (B) Schematic of the genomic DNA region containing the Syrian hamster *cad* promoter. Restriction digestion with *Bam*HI releases a 2.2-kb genomic fragment (dashed line) containing *cad* promoter sequences from −333 to +1867. The transcription start site is depicted by the closed bent arrow at +1. The location of the 555-bp probe for Southern blot analysis is indicated by the dotted line. (C) Southern blot analysis of immunoprecipitated DNA. After UV treatment and digestion with *Bam*HI, chromatin from 5×10^7 B5-4 cells was incubated with 2 μg of monoclonal antibody 8WG16 (anti-RNA polymerase II) and then with 2 μg of rabbit anti-mouse IgG-IgA-IgM (heavy plus light chain) polyclonal secondary antibody (2°) or with the secondary antibody alone. Immunoprecipitated complexes were processed and electrophoresed on an agarose gel for Southern blot transfer. The blot was probed with a 555-bp fragment complementary to *cad* promoter sequences from −333 to +222, as shown in panel B. DNA size markers are indicated on the right.

Although the UV cross-linking immunoprecipitation technique has been used successfully to study the interactions between transcription factors and their target promoters in *Drosophila* embryos (54), it has not been adapted for similar studies in mammalian cells. One limitation of this technique for use in the study of mammalian transcription factors has been the sensitivity of the assay. The mammalian genome is approximately 10-fold larger than the *Drosophila* genome, making detection of an immunoprecipitated gene fragment out of the entire population of DNA significantly more difficult. To alleviate this problem, we used a Syrian baby hamster kidney cell line, B5-4, which contains an amplification of the *cad* locus. The *cad* gene was amplified to approximately 50 copies in the B5-4 cell line through selection with the drug PALA, a specific inhibitor of the aspartate transcarbamylase activity of CAD (53). Because each amplicon is very large (approximately 500 kb), containing the entire *cad* gene as well as flanking DNA, the amplified copies of *cad* are maintained in their native chromosomal context. In addition, these cells grow readily in suspension culture to provide the large quantities of cells needed for the UV cross-linking assay. In the related adherent cell line BT-4, *cad* expression has been shown to be growth regulated, suggesting that the amplified genes contain all of the sequences necessary for correct regulation (36).

In *Drosophila* cells, RNA polymerase II is efficiently cross-linked to DNA by UV irradiation (26). Because RNA polymerase II is a good substrate for UV cross-linking and should be bound at the promoter of any actively transcribed gene, we chose to begin developing the UV cross-linking assay in mammalian cells by looking at the interaction between RNA polymerase II and the *cad* promoter in B5-4 cells. Following UV treatment and *Bam*HI digestion, B5-4 chromatin was immunoprecipitated with a mouse monoclonal antibody (8WG16) targeted against the C-terminal domain of eukaryotic RNA

polymerase II. As shown in Fig. 1B, *Bam*HI digested B5-4 chromatin is predicted to yield a 2.2-kb fragment of the *cad* promoter which includes the transcription start site (+1) as well as the consensus E box (+65). Southern blot analysis of 8WG16 immunoprecipitates demonstrates the presence of a 2.2-kb fragment which corresponds to the *cad* promoter (Fig. 1C, lane 3). Enrichment for the *cad* promoter is dependent on 8WG16, as immunoprecipitation in the presence of secondary antibody alone does not yield a 2.2-kb *cad* fragment (Fig. 1C, lane 2). As a control for efficient chromatin digestion and Southern transfer, a sample representing 0.01% of input chromatin prior to the addition of antibody was included on each gel (Fig. 1C, lane 1). The ability to easily detect the *cad* promoter fragment after immunoprecipitation with the RNA polymerase II antibody suggests that our assay is sensitive enough to study mammalian transcription factor-DNA interactions in vivo.

Max and USF are associated with the *cad* promoter in vivo. The E-box motif, CACGTG, is recognized by both USF and the Myc-Max heterodimer, as demonstrated by in vitro binding studies (11, 28). To determine which of these factors, if any, binds the chromosomal *cad* E-box element, we used the same in vivo cross-linking immunoprecipitation conditions as for the RNA polymerase II experiments. The immunoprecipitation step was carried out with antibodies to USF, Myc, and Max. Although these antibodies have previously been shown to efficiently immunoprecipitate their respective targets from human, mouse, and rat cells, their specificity for hamster proteins was uncertain. Western blot analysis of B5-4 nuclear extracts (Fig. 2A) demonstrates that the USF, Myc, and Max antibodies recognize hamster proteins approximately 44, 67, and 22 kDa in size, respectively, which correspond to the predicted molecular weights for the proteins. Therefore, the USF, Myc, and Max antibodies chosen for these experiments should specifically recognize and immunoprecipitate their target hamster immunogens.

We incubated UV-treated, *Bam*HI-digested chromatin with antibodies to USF, Myc, and Max. As before, immunoprecipitates were analyzed by Southern blotting and probing for the presence of the 2.2-kb *cad* promoter fragment. Two representative Southern blots, presented in Fig. 2B, show that antibodies to Max, Myc, and USF can immunoprecipitate the *cad* promoter fragment. We observed that anti-Max immunoprecipitates consistently contained enough of the 2.2-kb *cad* promoter fragment to yield a signal (similar in intensity to that seen in RNA polymerase II immunoprecipitated samples) easily detectable by Southern blotting. The consistency of the Max antibody to select for the *cad* promoter fragment is shown in Fig. 2B. In these experiments, a clear *cad* signal is detected in the Max immunoprecipitates from each of three independent preparations of cross-linked chromatin. A summary of the UV cross-linking immunoprecipitation data is presented in Table 1. Following several independent immunoprecipitation experiments performed on separate preparations of chromatin, we observed Max (as well as RNA polymerase II) associated with the 2.2-kb *cad* fragment in every trial. However, USF- or Myc-immunoprecipitated *cad* signals were sometimes undetectable, especially in cases in which the Max immunoprecipitated *cad* signal was faint. As shown in Table 1, USF-*cad* promoter and Myc-*cad* promoter interactions were identified in 80 and 30% of the experiments, respectively. In all of the experiments presented in Table 1, no *cad* fragment was detected in the absence of primary antibody. In addition, no signal was observed when the Southern blots were reprobed with *cad* sequences located upstream of the 2.2-kb *Bam*HI fragment, suggesting that immunoprecipitation is specific for the *cad* promoter (data not shown). Taken together, these results suggest that in vivo, the *cad* E box is bound by both Max, as detected in 10 of 10 experiments, and USF, as shown in 4 of 5 experiments. Since Myc is the only binding partner of Max which has been shown to be both involved in transactivation and present in growing cells, it is likely that Myc is bound to the *cad* promoter as a heterodimer with Max. We believe that the difficulty in detecting Myc-*cad* and USF-*cad* interactions may be due to less efficient immunoprecipitation by these antibodies.

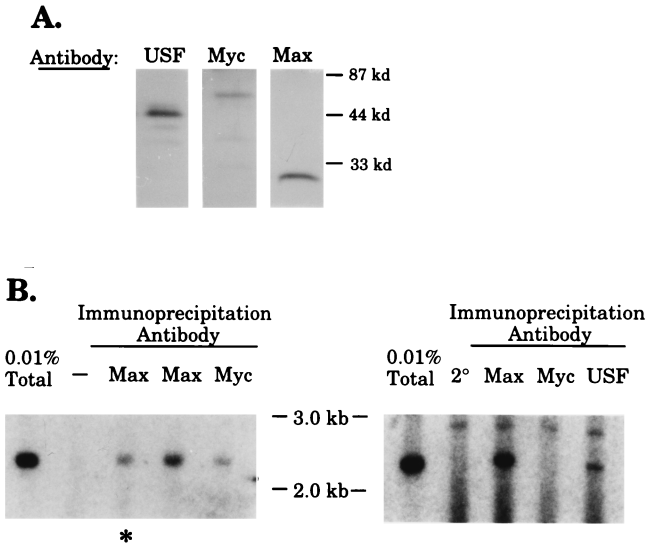

FIG. 2. The E-box-containing fragment of the *cad* promoter is immunoprecipitated by antibodies to Max, Myc, and USF. (A) Western blot analysis of B5-4 nuclear extracts. Nuclear extract from 1.3×10^6 B5-4 cells was resolved by SDS-PAGE and probed with 2 μg of rabbit polyclonal antibody to USF, Max, or Myc. Following incubation with a peroxidase-conjugated secondary antibody, proteins were detected by enhanced chemiluminescence. Kaleidoscope molecular weight standards (Bio-Rad) were electrophoresed next to nuclear extract samples. (B) Southern blot analysis of DNA immunoprecipitated by antibodies to Max, Myc, and USF. The asterisk indicates that the input chromatin was prepared from a different preparation of UV-cross-linked B5-4 cells than the other samples on the blot. UV-cross-linked and *Bam*HI-digested chromatin was incubated with 2 μg of anti-Max, anti-Myc, anti-USF, or anti-mouse secondary (2°) polyclonal antibody or in the absence of any antibody as indicated. Immunoprecipitated complexes were processed and run on an agarose gel for Southern blot transfer. The blot was probed with a 555-bp fragment complementary to *cad* promoter sequences from −333 to +222, as shown in Fig. 1. DNA size markers are indicated.

Sequences which flank the *cad* consensus E box can confer binding specificity. Although Myc-Max and USF can bind the *cad* E-box sequence in vivo, it was not clear whether binding of USF or Myc-Max resulted in equivalent transactivation of the

TABLE 1. Summary of results using UV cross-linking and immunoprecipitation to detect protein-*cad* promoter interactions

Immunoprecipitation antibody	Total expts[a]	Positive signal[b]
RNA polymerase II	3 (2)	3
Max	10 (6)	10
Myc	7 (6)	2
USF	5 (5)	4
No primary antibody	12 (6)	0

[a] Number of immunoprecipitations performed with each antibody. Numbers in parentheses indicate the number of separate preparations of UV cross-linked B5-4 chromatin used for each set of immunoprecipitation reactions.

[b] No. of experiments in which the 2.2-kb *cad* promoter fragment was detected with the indicated antibody (for examples, see Fig. 1 and 2).

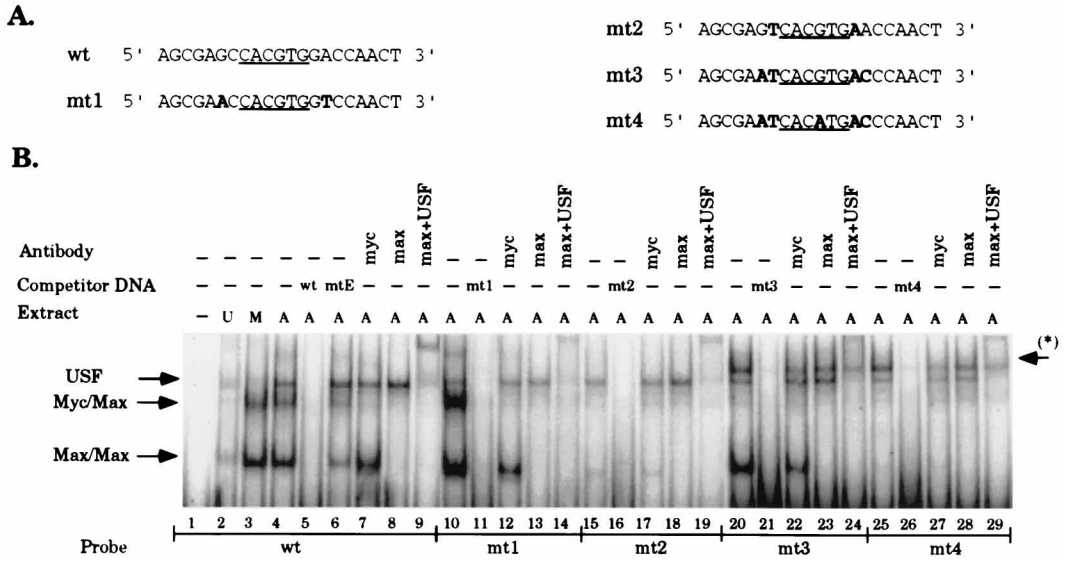

FIG. 3. Sequences flanking the core E-box element in the *cad* promoter determine Myc-Max and USF binding affinity. (A) Oligonucleotide sequences used as double-stranded probes in gel shift reactions. Sequence of the wild-type (wt) *cad* E-box oligonucleotide corresponds to the endogenous hamster *cad* promoter from nucleotides +55 to +75 relative to the transcription start site (21). The consensus E-box motif is underlined. Mutant oligonucleotides mt1 to mt4 contain changes in the nucleotides which immediately flank the *cad* E box. Boldface indicates nucleotides which differ from the wild-type *cad* sequence. (B) Gel shift analysis of wild-type and mutant *cad* sequences. End-labeled, double-stranded oligonucleotide probes shown in panel A were incubated with no protein (−), unprogrammed rabbit reticulocyte lysate (U) (which contains a small amount of endogenous USF and Max), reticulocyte lysate in which both Myc and Max mRNAs have been translated (M), or M plus HeLa nuclear extract (A). Where indicated, protein mixture A was preincubated with a 50-fold molar excess of unlabeled oligonucleotide prior to the addition of the indicated probe (mtE competitor oligonucleotide is equivalent to the wt sequence above except it contains a mutated E box, CCTGCAGG). Proteins were also preincubated with rabbit polyclonal antibodies to Myc, Max, USF, or combinations thereof prior to addition of labeled probe as indicated. Complex formation was monitored by electrophoretic fractionation in a nondenaturing polyacrylamide gel. Dried gels were exposed to PhosphorImager screens and scanned with ImageQuant software. Specific protein-DNA complexes are indicated by the arrows. The asterisk denotes an unidentified protein-DNA complex (see text).

cad promoter at the G_1/S-phase boundary. Using growth cycle-staged nuclear extracts, we have previously shown that the binding activity of both USF and Max-containing complexes increases on the *cad* E box as cells progress through late G_1 phase (43). To determine if both factors could regulate *cad* expression during the growth cycle, it was necessary to create promoter constructs which would preferentially bind Myc-Max and not USF, or vice versa. It has been suggested that the context of the E-box element with respect to the nucleotides which directly flank the core consensus is important for conferring specificity of factor binding (9). Therefore, we examined whether changing the context of the core CACGTG consensus in the *cad* promoter would allow for discrimination between Myc-Max and USF binding.

To study the binding properties of the *cad* E-box element and additional flanking mutants, we used gel mobility shift assays. A double-stranded oligonucleotide which corresponds to sequences from +55 to +75 of the endogenous hamster *cad* promoter was created (Fig. 3A, wt). This oligonucleotide contains the core consensus E box (CACGTG) flanked by native sequences. We made four additional oligonucleotides which contain mutations in either the nucleotides which immediately flank the consensus E box or within the core E-box sequence (Fig. 3A, mt1 to mt4). These particular mutations were chosen for our analysis because they have been shown to influence Myc-Max and USF binding to other E-box elements (9). Using the same gel shift conditions, each *cad* oligonucleotide was tested for the ability to bind to USF and Myc-Max heterodimers.

Work from our laboratory, as well as others, has demonstrated that both USF and Max binding can be detected in nuclear extracts. In contrast, the binding activity of Myc-Max is difficult to assay with nuclear extracts from mammalian cells (20, 43). Therefore, to study binding of both USF and Myc-Max, we chose to use HeLa nuclear extract as a source of USF and in vitro-translated proteins as a source of Myc and Max. We combined these two sources of protein so that binding of USF and Myc-Max could be analyzed together under the same conditions. When the wild-type *cad* E box is used as a probe for protein binding, three different protein complexes are observed (Fig. 3B, lane 4). These complexes are specific to the *cad* E box, as preincubation with a 50-fold excess of unlabeled probe eliminates all binding (lane 5). Cold competition with an identical oligonucleotide containing a mutant E-box sequence (CACGTG to CTGCAG) has no effect on binding (lane 6). The three binding complexes were identified as USF, Myc-Max, and Max-Max by antibody competition. Addition of an anti-Myc antibody to the gel shift reaction specifically disrupts the middle complex (lane 7), and the anti-Max antibody disrupts both the bottom complex and the middle complex (lane 8), indicating that the bottom complex is Max-Max homodimers and the middle complex is Myc-Max heterodimers. The remaining upper complex can be supershifted by incubation with an anti-USF antibody (lane 9). These results demonstrate that in vitro, the natural context of the wild-type *cad* E box allows for the binding of either USF or Myc-Max transcription factors. Therefore, for the wild-type *cad* E box, the in vitro binding assay faithfully reproduces the binding results that we obtained using in vivo cross-linking.

We next tested the ability of each protein to form a gel shift complex on the *cad* E box oligonucleotides which contain flanking sequence mutations. We observed that the binding pattern is influenced by the nucleotides which flank the consensus CACGTG. As shown in Fig. 3B, each mutant probe displays a unique gel shift pattern. mt1, which has both nucleotides at the −5 and +5 flanking positions mutated to an A

and a T, respectively, has a pattern of binding similar to that of the wild-type probe (lane 10). Different results were obtained when the oligonucleotides corresponding to mt2, mt3, and mt4 were used as gel shift probes. Each of these E-box-flanking mutants has the immediate flanking nucleotides altered to a T and an A at positions −4 and +4, respectively, with additional changes in mt3 and mt4. The mutations present in mt2, mt3, and mt4 decrease binding of Myc-Max heterodimers to each probe, as shown by the loss of this gel shift complex below detectable levels (although mt3 does retain the ability to bind to Max homodimers). However, USF complex formation is not affected, as seen by comparing lanes 15, 20, and 25 to lanes 19, 24, and 29. In addition to reducing Myc-Max binding, mt3 and mt4 bind a new complex which is not competed by antibodies to Myc, Max, and USF. This result suggests that the new nucleotides at positions −5 and +5 present in mt3 and mt4 create a binding site for another cellular factor. We believe that AP1 is a good candidate for binding to mt3 and mt4 because of the homology between these oligonucleotides and the AP1 consensus sequence (3); however, we have not yet tested this possibility. In summary, our in vitro studies suggest that the wild-type *cad* E box and mt1 contain favorable binding sites for Myc-Max; in contrast, mt2, mt3, and mt4 bind Myc-Max very poorly.

Growth-dependent G_1/S-phase transcriptional regulation of the *cad* promoter is abolished by E-box-flanking nucleotides which reduce Myc-Max binding in vitro. Both the in vivo cross-linking and in vitro gel shift assays show that the *cad* E box in its natural context may be bound by either Myc-Max or USF, suggesting that either of these transcription factors may be responsible for *cad* G_1/S-phase transcriptional activation. Since we have shown that nucleotides flanking the E-box consensus sequence can influence Myc-Max and USF binding, we next examined how flanking sequence mutations would affect E-box-mediated transcriptional regulation of *cad* expression in vivo. We constructed *cad* promoter reporter plasmids which contained the wild-type E box or an E box having mutations in the flanking sequence. All constructs were cloned upstream of the luciferase cDNA (Fig. 4A). To study promoter activity during the growth cycle, growing NIH 3T3 cells were transiently transfected with reporter plasmids and serum starved until quiescent. Upon addition of serum to the culture medium, quiescent cells synchronously progress through the first cell cycle, as judged by flow cytometric analysis (data not shown), allowing for assessment of growth-induced promoter activity in early S phase.

Prior work from our laboratory has demonstrated that the minimal *cad* promoter from −81 to +26 (which is sufficient for accurate basal transcription) can be converted to a growth-regulated promoter by the addition of E-box-containing sequences (+55 to +75) cloned directly downstream of +26. Based on these results, we chose to use the −81/+26[wt] promoter reporter construct as a positive control for this experiment. As shown in Fig. 4B, the −81/+26[wt] construct was growth regulated, showing the same S-phase induction as previously reported (43). The −81/+26 minimal promoter was used as a negative control. This construct is unresponsive in our serum starvation-stimulation assay, reproducing the earlier published results as well.

To study the effect on *cad* S-phase regulation, each E-box-flanking mutant was cloned downstream of the minimal *cad* promoter and tested in the transient transfection, serum starvation-stimulation assay. As demonstrated in Fig. 4B, addition of the mt1 E box to the −81/+26 promoter results in a 10-fold increase in S-phase reporter activity. This result indicates that the mt1 oligonucleotide, which binds preferentially to the Myc-Max heterodimer in vitro, can confer growth-induced transcriptional activation to the minimal promoter. In contrast, the other E-box-flanking mutants did not confer growth regulation to the −81/+26 *cad* promoter. Adding E-box mutants mt2, mt3, and mt4 to the minimal promoter resulted in only a twofold increase in S-phase activity, the same induction observed for the −81/+26 minimal promoter which lacks an E box. The oligonucleotides mt2, mt3, and mt4 all bind well to USF but not to Myc-Max. In addition, we note that mt3, which also binds to Max-Max homodimers, cannot regulate *cad* expression.

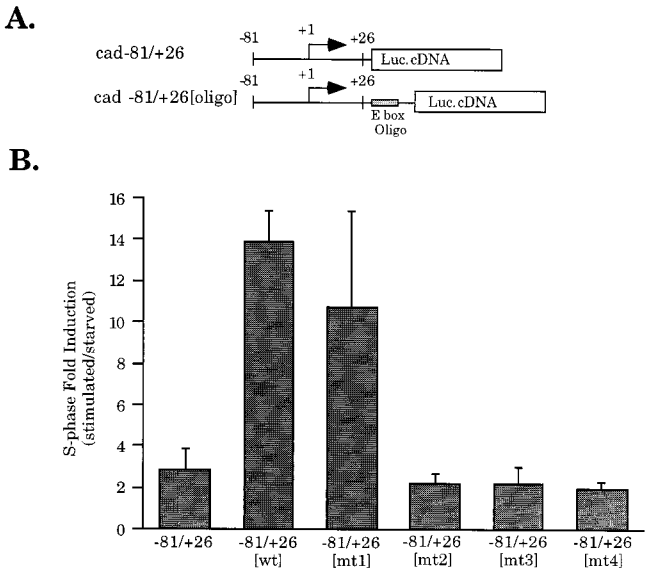

FIG. 4. Correlation between Myc-Max binding and *cad* G_1/S-phase transcriptional activation. (A) Schematic representation of *cad* promoter reporter constructs used in transfection assays. Hamster *cad* promoter sequences were fused to the luciferase (Luc.) cDNA. Oligonucleotides corresponding to sequences from +55 to +75 of the *cad* promoter and mutations thereof were inserted at +26 of the *cad* promoter reporter construct as shown. The sequences of the inserted oligonucleotides are presented in Fig. 3A. (B) Graphical representation of the average S-phase induction of transiently transfected *cad* promoter reporter constructs. NIH 3T3 cells were transiently transfected with the indicated *cad* reporter constructs. Following transfection, cells were incubated in starvation medium (0.5% serum) for 48 h. In parallel cultures, starvation medium was replaced with stimulation medium (10% serum) for 16 h, and cells were harvested for luciferase activity. The fold induction at 16 h (S phase) is reported as the ratio of luciferase activity from serum-stimulated cells to the activity of the same construct in serum-starved cells. Data presented were obtained from 3 to 10 independent experiments, using multiple DNA preparations.

In this experiment, we were not able to use the UV cross-linking procedure to verify that the E-box-flanking mutants mt2, mt3, and mt4 bind USF, but not Myc-Max, in transfected cells. This assay is not currently sensitive enough to detect protein binding to endogenous single-copy genes or to exogenous low-copy-number plasmids. However, based on the results of the gel shift assays, our observations provide evidence that the binding of Myc-Max heterodimers to the *cad* E box is necessary for S-phase transcriptional activation and indicate that the sequences flanking the core E-box consensus are important for specifying proper regulation of *cad* expression.

The consensus initiator is not required for E-box-mediated activation of the *cad* promoter. We were interested in identifying which *cis* elements in the *cad* minimal promoter allow for E-box-mediated transcriptional activation by Myc-Max. The minimal *cad* promoter consists of two Sp1 sites, a binding site for an uncharacterized cellular factor named HONK, and a consensus initiator element (33). In other promoters, Myc and

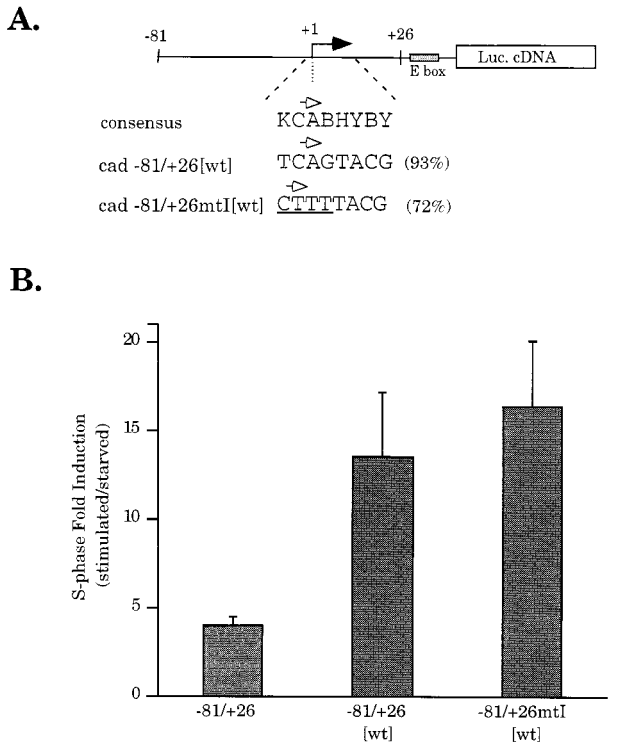

FIG. 5. A consensus initiator element is not required for E-box-mediated transcriptional activation of the *cad* promoter. (A) Schematic representation of *cad* promoter reporter constructs used in transfection assays. Initiator sequences are presented below the schematic in capital letters, and underlined sequences indicate changes from the wild-type sequence. Consensus initiator sequences are as follows: K is G or T, B is C, G, or T, H is A, C, or T, and Y is C or T. The highest percent match (shown in parentheses) to the consensus sequence was determined as in reference 14 for transcription initiation at the base corresponding to the open-headed arrow. Luc., luciferase. (B) Graphical representation of the average S-phase induction of transiently transfected *cad* promoter reporter constructs. Transfections were performed as described in the legend to Fig. 4B. Data presented were obtained from three independent experiments.

USF have been shown to influence transcription through the initiator element (35). To address the possibility that the *cad* initiator is a required element for activated transcription, we designed a *cad* promoter reporter construct in which consensus initiator sequences have been replaced with random sequences (Fig. 5A). With this mutation, the match to a consensus initiator element was decreased from 93% (wild-type sequences, −81/+26[wt]) to 72% (mutant sequences, −81/+26mtI[wt]). Since initiator sequences with less than an 81.3% match to the consensus are no longer considered significant (14), we were confident that the mutated *cad* promoter no longer contained a functional initiator. We have shown previously that this initiator mutation does not disrupt basal transcription from the *cad* minimal promoter in vivo (33). To examine *cad* G_1/S-phase transcriptional activation in the absence of the consensus initiator element, serum starvation-stimulation assays were performed with the initiator mutant construct (−81/+26mtI[wt]) and the wild-type *cad* promoter (−81/+26[wt]). As shown in Fig. 5B, we observe a 12- to 15-fold induction of *cad* promoter activity in S phase, both in the presence and in the absence of a consensus initiator element. These results indicate that the consensus initiator is not a required *cis* element for E-box-mediated transcription from the *cad* minimal promoter.

DISCUSSION

We have shown previously that a consensus E-box element in the *cad* promoter is essential for activated transcription at the first G_1/S-phase boundary after quiescent cells are stimulated to reenter the cell cycle. Therefore, the focus of this study was to discern which E-box-binding factor(s) is associated with *cad* expression. The E-box-binding proteins, like most DNA-binding transcription factors, belong to a multigene family in which all members have the ability to bind in vitro to a common consensus DNA element. This lack of binding specificity might be interpreted as evidence that E-box family members are functionally redundant. However, in vivo studies do not support this conclusion. For example, enforced expression of Myc, but not USF, can cooperate with Ras to transform cells (37). This observation supports a model in which discrimination between Myc and USF is achieved at certain target genes in vivo. We now show that the *cad* promoter can be regulated by Myc but not by USF.

To begin our studies, we considered that the lack of discrimination between family member binding in vitro may not accurately reflect gene regulation in cells. It is possible that selective binding of transcription factors is actually achieved in cells under physiological conditions but that this selectivity is lost in cellular extracts. Selectivity may also be lost in transfection assays. In the search for bona fide Myc target genes, researchers have relied on overexpression of Myc and USF to assay for effects on gene expression. One consequence of Myc overexpression is that it may result in binding at sites not normally occupied in the presence of low levels of Myc. In contrast, we were interested in determining whether Myc or USF binds at a specific E-box-containing promoter under physiological conditions. Toward this goal, we used an in vivo UV cross-linking and immunoprecipitation procedure to examine protein binding to the *cad* promoter. The advantages of this assay are twofold. First, we have not altered the amount or timing of expression of any transcription factor in the cell. Second, we are examining the binding of cellular factors to a promoter located in its native chromosomal context. Using this technique, we observed that antibodies to Max, Myc, and USF immunoprecipitated the *cad* promoter. Due to differences in protein cross-linking efficiency and immunoprecipitation efficiency, we cannot directly compare the in vivo binding affinities of Myc, Max, and USF for the *cad* E box. However, it is clear that in the cell, each of these proteins can bind to the *cad* promoter. We do not believe that differential timing of DNA binding provides specificity, since growth-staged extracts show little binding activity of any protein to the *cad* E box in G_0-phase nuclear extracts and considerable binding of both USF and Max-containing complexes in late-G_1-phase extracts (43).

Our results suggest that discrimination at the *cad* E box in vivo occurs not through timing or specificity in DNA binding but at a subsequent step. To examine this possibility, we compared the ability of Myc or USF to activate the *cad* promoter in S phase. Since both factors bind the endogenous *cad* E box in vivo, we created reporter constructs which discriminate between Myc-Max and USF binding in vitro. Using these constructs in transfection assays, we observed a correlation between decreased Myc-Max binding at the *cad* E box and the loss of S-phase activation. These results suggest that Myc and USF are not equivalent in their abilities to influence *cad* regulation. In Fig. 6, we propose a model in which, as cells progress from quiescence into S phase, Myc-Max competes with USF for binding to the *cad* E box. Although binding of either complex can occur, we propose that activated transcription is achieved only by Myc-Max, not by USF. One important

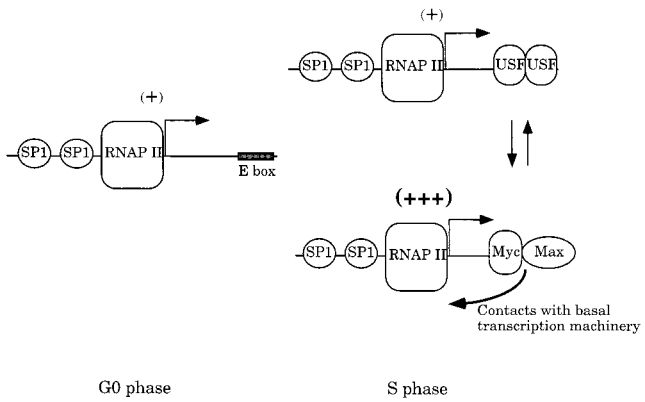

FIG. 6. Discrimination between regulation by Myc and USF is determined by core promoter elements. As described in the text, our in vitro and in vivo data suggest that there is no selectivity in binding of USF versus Myc-Max to the endogenous *cad* promoter. Our results suggest that no complexes are bound to the promoter in quiescent cells and that both Myc-Max and USF can bind in S phase. However, activated transcription in S phase is achieved only when Myc-Max is bound, not when USF is bound. We propose that transcriptional activation of the core *cad* promoter requires specialized contacts between Myc-Max and the basal transcription machinery. RNAP, RNA polymerase.

aspect of this model is that activation of E-box-containing genes is influenced both by the core promoter and by levels of E-box-binding proteins in the cell. All of our studies are performed with quiescent cells that are stimulated to reenter the cell cycle. We have shown that in NIH 3T3 cells, the levels of c-Myc protein peak at 8 h after serum stimulation (43). We have not examined the regulation of *cad* during the proliferative cell cycle, which, in contrast to the growth cycle, displays constitutive levels of c-Myc protein (reviewed in reference 39).

Selective activation of certain E-box-containing promoters, such as the *cad* promoter, may explain why Myc, but not USF, can transform cells. However, although USF cannot activate the *cad* promoter, it can displace Myc-Max. This may be why USF displays a growth-inhibitory phenotype when it is expressed with Myc in a Ras cotransformation assay (37).

We have shown that Myc, but not USF, can activate the *cad* promoter in S phase of the growth cycle; however, we do not yet understand how differential activation is achieved. α-*prothymosin* is another gene where discrimination between Myc and USF occurs through differential activation. In the case of α-*prothymosin*, the distance of the E box from the transcription start site is critical for specificity because Myc, but not USF, can influence gene expression from distal enhancer positions (19). Since the *cad* E box is proximal to the transcription start site, discrimination between Myc and USF activity is not achieved by the position of the binding site and therefore must occur through an alternative mechanism. One possibility is that *cis* elements in the core *cad* promoter are required for specificity of Myc activation versus USF activation. The minimal *cad* promoter has two Sp1 sites, a binding site for an uncharacterized protein called HONK, and a consensus initiator element (33). Since Myc and USF can influence transcription through the initiator, we have mutated this element in the *cad* minimal promoter. We observed no change in *cad* S-phase activity, suggesting that the initiator is not required for E-box-mediated activation of the core *cad* promoter. Because Sp1 is essential for basal transcription, it is not possible to easily examine the role of Sp1 in *cad* S-phase activation through deletion of this element. An alternative possibility is that differential activation is influenced by the absence of certain elements in the core *cad* promoter. For example, *cad*, like many housekeeping genes, does not have a TATA box-like sequence in its promoter. Recently, it has been demonstrated the presence of a TATA box is required for maximal activation by USF2 (38). Although this study was performed with heterologous promoters, it does suggest that promoter context may influence USF activity.

It is clear that while both Myc and USF are transcription factors, they have different requirements to achieve activation of target promoters. Although USF and Myc have homologous DNA-binding domains, these factors have dissimilar transactivation domains which may enable Myc and USF to interact with different cellular proteins. The transactivation domain of Myc has been shown to contact the TATA-binding protein subunit of TFIID and the RAP74 subunit of TFIIF (29, 40). Evidence for USF suggests that interaction with TFIID may occur through the TATA-binding protein-associated factor $TAF_{II}55$ (18). These interactions suggest a functional difference between Myc and USF which may be critical for transactivating different types of promoters. Myc has also been shown to bind the p107 protein in vivo (7). Although the exact nature of this interaction is not fully understood, it is believed that a p107-associated kinase activity, cyclin A–cyclin-dependent kinase, is important for regulating Myc function. One possibility is that this kinase activity mediates Myc function through phosphorylation of a component of the basal transcription machinery. For example, phosphorylation of the C-terminal domain (CTD) of RNA polymerase II is associated with promoter clearance. Although CTD phosphorylation can be achieved by components of the basal machinery (22, 48), it may be enhanced by additional cellular kinases, such as the Myc–p107–cyclin A–cyclin-dependent kinase complex. We have shown that the CTD of RNA polymerase II is required for *cad* transcription (15). Therefore, in the context of a promoter which requires the CTD for efficient transcription, Myc and USF may have different activities.

In conclusion, we have shown that the *cad* promoter is an in vivo target for Myc and USF binding under physiological conditions. We have shown that activated transcription from the *cad* promoter is specifically achieved by the Myc-Max transcription factor and not by USF. These results suggest that *cad* represents a class of Myc target genes where discrimination between E-box-binding proteins is achieved not by the sequence or location of the E box but rather by the core promoter.

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