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Activation of the Murine Dihydrofolate Reductase Promoter by E2F1

A REQUIREMENT FOR CBP RECRUITMENT*

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The E2F family of heterodimeric transcription factors plays an important role in the regulation of gene expression at the G_1/S phase transition of the mammalian cell cycle. Previously, we have demonstrated that cell cycle regulation of murine dihydrofolate reductase (*dhfr*) expression requires E2F-mediated activation of the *dhfr* promoter in S phase. To investigate the mechanism by which E2F activates an authentic E2F-regulated promoter, we precisely replaced the E2F binding site in the *dhfr* promoter with a Gal4 binding site. Using Gal4-E2F1 derivatives, we found that E2F1 amino acids 409–437 contain a potent core transactivation domain. Functional analysis of the E2F1 core domain demonstrated that replacement of phenylalanine residues 413, 425, and 429 with alanine reduces both transcriptional activation of the *dhfr* promoter and protein-protein interactions with CBP, transcription factor (TF) IIH, and TATA-binding protein (TBP). However, additional amino acid substitutions for phenylalanine 429 demonstrated a strong correlation between activation of the *dhfr* promoter and binding of CBP, but not TFIIH or TBP. Finally, transactivator bypass experiments indicated that direct recruitment of CBP is sufficient for activation of the *dhfr* promoter. Therefore, we suggest that recruitment of CBP is one mechanism by which E2F activates the *dhfr* promoter.

Regulation of eukaryotic gene expression requires sequence-specific DNA binding transcription factors that contain at least two essential domains: a DNA binding domain that directs the protein to its target promoter, and a domain that facilitates transcriptional regulation by contacting a variety of cellular proteins (1, 2). Proteins contacted by DNA binding transcription factors include general transcription factors (TF)[1] such as TFIIA (3), TFIIB (4), TFIID (5–7), TFIIF (8, 9), and TFIIH (10); coactivators such as the CREB-binding protein (CBP) (11), GCN5 (12), ADA2 (13, 14), and components of the SWI/SNF complex (15, 16); and corepressors such as retinoblastoma (Rb) (17), Sin3 (18, 19), and KRAB-associated protein-1 (KAP-1) (20). These interactions are thought to facilitate transcriptional regulation by directly altering the recruitment and/or activity of RNA polymerase II transcription complexes, or by modifying the chromatin structure of a gene (for a recent review, see Ref. 21). Several studies provide evidence indicating that different gene promoters require different protein-protein interactions for activation. For example, recruitment of the TATA-binding protein (TBP) appears to be important for TATA-containing, but not TATA-less promoters (22). Additionally, the herpes simplex virus transactivator VP16 is a more robust activator of TATA-containing promoters than TATA-less promoters, whereas Sp1 shows no TATA preference, suggesting functional redundancy of the TATA element and Sp1, but not VP16 (23, 24). In addition, the degree to which a transcription factor contributes to activation of a promoter can be influenced by the presence of other bound transactivators, which can provide either additive or redundant functions. Synergistic activation by two or more transactivators can be the result of multiple protein contacts that stimulate distinct stages of transcription (25). For example, the human immunodeficiency virus Tat transactivator protein stimulates transcriptional elongation and can synergize with Sp1 (which stimulates initiation, but not elongation), but not with VP16, p53, or E2F1 (all of which stimulate elongation) (25). Therefore, the apparent potency of a particular transactivation domain and the importance of specific protein-protein interactions may vary considerably in different promoter contexts.

We wish to understand the mechanisms by which site-specific DNA binding proteins activate the murine *dhfr* promoter, which contains four Sp1 binding sites and an E2F binding site that overlaps the start site of transcription. Our studies indicate that the Sp1 binding sites are critical for basal promoter activity, while the E2F binding site is required for growth-regulated transcription (26–29). *In vivo* footprinting analysis of the hamster *dhfr* promoter demonstrated that protein binding to one strand of the E2F element correlates with the increase in promoter activity in late G_1 and early S phase, suggesting that an E2F family member activates *dhfr* transcription in S phase (30). In support of this hypothesis, we have shown that the E2F site is critical for high levels of activity from the murine *dhfr* promoter in S phase (29). The focus of our current studies is to determine the molecular mechanisms by which proteins that

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[1] The abbreviations used are: TF, transcription factor; CBP, CREB-binding protein; CREB, cAMP response element-binding protein; TBP, TATA-binding protein; GST, glutathione *S*-transferase; DHFR, dihydrofolate reductase; Rb, retinoblastoma; ACB, Affinity Chromatography Buffer; HDAC, histone deacetylase.

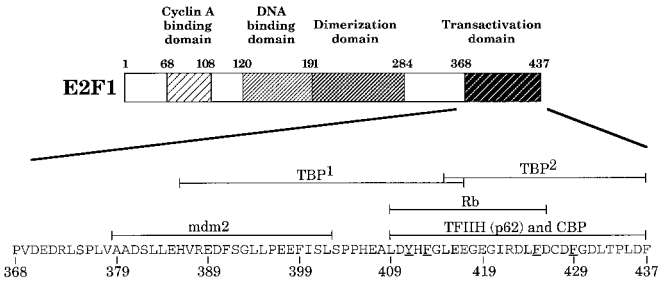


FIG. 1. **The E2F1 transactivation domain binds several cellular proteins.** Schematic of the human E2F1 protein depicting previously characterized functional domains (17, 38, 39, 46, 53). The transactivation domain is magnified to indicate the binding domains for mdm2, Rb, the p62 subunit of TFIIH, CBP, and the two independent binding domains for the TBP. *Underlined* amino acid residues were mutated in this study.

bind to the E2F site regulate *dhfr* transcription.

The E2F family of transactivator proteins mediates both transcriptional activation and repression through interactions with multiple target proteins. Eight members of this family have been identified: six E2F proteins (E2F1 to E2F6) and two DP proteins (DP1 and DP2). The E2F and DP proteins bind to DNA as a heterodimer to form a functional E2F/DP complex (for a recent review, see Ref. 31). The transcriptional repression function of E2F/DP is attributed to the interaction between E2F family members and the Rb family of transcriptional repressor pocket proteins. E2F1, E2F2, and E2F3 bind to the Rb protein, E2F4 binds to Rb and the related p107 and p130 proteins, and E2F5 binds to p130 (for recent reviews, see Refs. 31 and 32). E2F6 (or EMA) does not bind pocket proteins, but is believed to contain an inherent repressor domain (33, 34). The pocket proteins are thought to repress transcription in G_0 and early G_1 phase cells by recruiting histone deacetylases to promoters and by blocking E2F protein-protein interactions required for activation of transcription (35–39). However, as cells approach the G_1/S phase boundary, the pocket proteins are phosphorylated and released from the E2F heterodimer, allowing E2F to function as a transcriptional activator. The activation functions of E2F/DP are mediated by a potent C-terminal transactivation domain in the E2F subunits.

We, and others, have previously shown that several of the E2F family members can activate the *dhfr* promoter when overexpressed using transient or integrated E2F expression constructs (28, 40, 41). Additionally, using a formaldehyde cross-linking procedure, we have recently shown that E2F1, E2F2, E2F3, E2F4, and E2F5 can all bind to the *dhfr* promoter *in vivo*.[2] Furthermore, we have demonstrated that Gal4 fusion proteins containing the transactivation domains of E2F1, E2F3, E2F4, and E2F5 activate a *dhfr* promoter construct containing a Gal4 binding site in precise replacement of the E2F binding site (29).[3] The *in vivo* binding data, the promoter activation experiments, and the fact that all of the E2F transactivation domains are very similar, suggests that all of the E2F family members may be *bona fide* activators of the *dhfr* gene. However, the E2F1 transactivation domain is the best characterized (Fig. 1), and therefore we have used E2F1 in our current experiments designed to determine the mechanism by which E2F family members activate the *dhfr* promoter.

Previous analyses of the E2F1 transactivation domain have employed Gal4 fusion proteins and reporter constructs that have randomly positioned single or multiple Gal4 binding sites cloned upstream of minimal promoters whose basal activity is mainly mediated by a TATA box. Due to the abundance of cellular E2F proteins, the use of Gal4 fusion proteins is required for the functional analysis of a specific E2F family member. However, most E2F-regulated promoters, including *dhfr*, are TATA-less promoters whose basal activity is conferred by transcriptional activators such as Sp1 and CCAAT binding proteins (42). Thus, prior functional analyses of the E2F1 transactivation domain may not accurately reflect the role of protein-protein interactions in the context of an authentic E2F-regulated cellular promoter. In support of this hypothesis, we have shown that, unlike E2F-mediated activation of synthetic promoters, activation of *dhfr* transcription is dependent on the position of the E2F binding site proximal to the transcription start site (29). Therefore, we have undertaken a functional analysis of E2F1 using an authentic E2F-regulated promoter, and present evidence that E2F1 recruitment of CBP is critical for activation of *dhfr* transcription.

MATERIALS AND METHODS

Plasmids—Standard cloning techniques were used for all plasmid constructions (43). The inserts for various E2F1 fusion proteins were made by polymerase chain reaction with the following primers: primer 1, 5′-CATAGAATAAGTGCGACATCATCATCGG-3′; primer 2, 5′-TAGTGGATCCTAGAAGCCCTGTCAGAAATCCAGGGGGGGTGAGGTCCCCAAAGTCACAGTCGGCGAGGT-3′; primer 3, 5′-TAGTGGATCCTAGAAGCCCTGTCAGAAATCCAGGGGGGGTGAGGTCCCCAGCGTCAC-3′; primer 4, 5′-TAGTGGATCCTAGAAGCCCTGTCAGAAATCCAGGGGGGGTGAGGTCCCCATAGTCAC-3′; primer 5, 5′-TAGTGGATCCTAGAAGCCCTGTCAGAAATCCAGGGGGGGTGAGGTCCCCTAAGTCAC-3′; primer 6, 5′-TAGTGGATCCTAGAAGCCCTGTCAGAAATCCAGGGGGGGTGAGGTCCCCATCGTCAC-3′; primer 7, 5′-TAGTGGATCCTAGAAGCCCTGTCAGAAATCCAGGGGGGGTGAGGTCCCCAGCGTCACAGTCGGCGAGGT-3′; primer 8, 5′-CCCGGATCCCTCGACGCCCACTTCG-3′; primer 9, 5′-CCCGGATCCCTCGACTACCACGCCG-3′; primer 10, 5′-CCCGGATCCTCAGAAATCCAGGGGGGT-3′.

For the construction of GST-E2F1 and Gal4-E2F1 expression plasmids, the appropriate polymerase chain reaction fragment was digested with *Bam*HI and cloned into the *Bam*HI site of pGEX-4T-1 (Amersham Pharmacia Biotech) or pBXG-1 (gift from Mark Ptashne). Both the GST and Gal4(1–147) versions of the following E2F1 constructs were made using the indicated primers and the E2F1 plasmid pBXG-1/E2F1(409–437) as template (39): E2F1(409–437)F425A, primers 1 and 2; E2F1(409–437)F429A, primers 1 and 3; E2F1(409–437)F429Y, primers 1 and 4; E2F1(409–437)F429L, primers 1 and 5; E2F1(409–437)F429D, primers 1 and 6; E2F1(409–437)F425A/F429A, primers 1 and 7. The Gal4 version of E2F1(409–437)Y411A was made using primers 8 and 10, and the plasmid pCMV-E2F1YA411 as template (44). The GST and Gal4 versions of E2F1(409–437)F413A were made using primers 9 and 10, and the plasmid pCMVFA413 as template (44). The GST and Gal4 versions of E2F1(409–437)3ΔF contain the triple alanine substitution F413A/F425A/F429A and were made using primers 1 and 7 with the E2F1 plasmid pBXG-1/E2F1(409–437)F413A as template. All other Gal4 and GST fusion protein expression plasmids used in these studies have been described previously and are referenced accordingly in the text. Gal4-E2F1ΔRb and Gal4-E2F1Δmdm2 have been described previously as Gal4-E2F1(368–437)(d418–422) and Gal4-E2F1(380–437)DF1 (45, 46). To construct Gal4-p62, a human p62 *Bam*HI/*Sal*I fragment from pAS1/p62 (gift from Errol Friedberg) was cloned into *Bam*HI/*Sal*I-digested pSG424 (47). The DHFRGal4 reporter construct contains *dhfr* promoter sequence from −356 to +20, with the E2F binding element precisely replaced with a Gal4 binding element, cloned upstream of the luciferase cDNA in the vector pAAlucA (29). The pG5TI and pMaeΔE2F reporter constructs have been described previously (27, 48).

Cell Culture and Transfection—NIH 3T3 cell cultures were maintained and calcium phosphate transfections were performed as described previously (29). Briefly, 1 day prior to transfection, 1.25×10^5 cells were seeded into 60-mm diameter dishes. Each dish of cells was transfected with 5 μg of reporter construct, 5 μg of Gal4-E2F1 expression construct, and 5 μg of sonicated salmon sperm DNA (Sigma). For the transfection of Gal4-CBP, Gal4-TBP, and Gal4-p62, 10–20 μg of Gal4 fusion expression constructs were used without the addition of salmon sperm DNA. Cells were incubated in growth medium for 40–48 h before harvesting, and total cell lysates were assayed for luciferase activity. Each transfection was repeated at least four times with duplicate samples and multiple DNA preparations.

[2] J. Wells and P. J. Farnham, unpublished data.
[3] C. J. Fry and P. J. Farnham, unpublished data.

Protein Affinity Chromatography—HeLa cell nuclear extract was prepared from frozen cells as described previously (49), and was dialyzed against Affinity Chromatography Buffer (ACB) (10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) containing 0.5 mM phenylmethylsulfonyl fluoride and 0.1 M NaCl. GST fusion proteins were prepared and immobilized on Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as follows. Overnight cultures of *Escherichia coli* strain DHF5α transformed with GST or GST-E2F1 expression plasmids were diluted 1:10 in 1 liter of fresh Luria Broth + ampicillin (50 μg/ml) and grown at 30 °C to an A_{600} between 0.5 and 1.0 before adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM. After 2–3 h of additional growth at 30 °C, cells were pelleted by centrifugation for 5 min at 5,000 rpm in a Beckman model J2–21 centrifuge (JA-17 rotor). Cell pellets were resuspended in 10 ml Buffer A (20 mM Tris-HCl (pH 7.4), 0.2 mM EDTA, 1 mM dithiothreitol, 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1× complete protease inhibitor (Roche Molecular Biochemicals), and lysed by mild sonication. Cellular debris was removed by centrifugation for 20 min at 11,500 rpm. Glycerol was added to the supernatant (to 10% final concentration), which was then frozen in liquid nitrogen and stored at −80 °C. *E. coli* GST lysates (precleared by centrifugation) were incubated with prewashed Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 2 h at 4 °C. The Glutathione 4B was then washed three times with 10 volumes of ACB containing 0.1 M NaCl, and stored at 4 °C as a 50% slurry in ACB containing 0.1 mM NaCl and bovine serum albumin (1 mg/ml). For the affinity chromatography experiments, 60 μl of GST-protein affinity columns (4 μg of GST fusion protein/μl of Glutathione-Sepharose 4B) were prewashed with eight column volumes of ACB containing 1 M NaCl and equilibrated with eight column volumes of ACB containing 0.1 M NaCl. 1 mg of HeLa nuclear extract was applied to the columns, which were then washed with 10 column volumes of ACB containing 0.1 M NaCl, and eluted with four column volumes of ACB containing 1% SDS. Eluates were analyzed by Western blot analysis with anti-p62 mAb3c9 (50), anti-CBP sc-583 (Santa Cruz), and anti-TBP 1TBP18 (gift from Nancy Thompson) antibodies. Autoradiograms were scanned and the signals were quantitated using ImageQuant version 4.2a (Moleular Dynamics). Each binding experiment was repeated at least four times.

RESULTS

A Core E2F1 Transactivation Domain Can Activate the dhfr Promoter—To investigate the activation of *dhfr* transcription by E2F1, we have precisely replaced the E2F binding site in the murine *dhfr* promoter with a Gal4 binding site to create a Gal4-responsive *dhfr* promoter (Fig. 2*A*). We have previously demonstrated that, like activation of the *dhfr* promoter by endogenous E2F, the activation of DHFRGal4 by a Gal4 fusion protein containing the E2F1 transactivation domain is dependent on the position of the Gal4 binding site proximal to the transcription start site, suggesting that Gal4-E2F1 and endogenous E2F activate the *dhfr* promoter through similar mechanisms (29). Using the DHFRGal4 promoter, and Gal4-E2F1 fusion proteins, we have now determined the minimal E2F1 transactivation domain and protein-protein interactions required for transcriptional activation of the *dhfr* promoter. Previous studies have implicated mdm2 and Rb in E2F1-mediated transactivation (46, 51, 52). To determine if recruitment of mdm2 or Rb is critical for activation of the *dhfr* promoter, we cotransfected NIH 3T3 cells with the DHFRGal4 reporter construct and either wild-type Gal4-E2F1 or Gal4-E2F1 expression constructs containing mutations that have been shown to abolish mdm2 or Rb binding (45, 46). At 2 days post-transfection, cells were harvested and assayed for luciferase activity. The activation of DHFRGal4 by the Gal4-E2F1 proteins is shown as percentage of activation, defining the activity of the wild-type Gal4-E2F1 constructs as 100% activation (Fig. 2). We found that the wild-type E2F1 transactivation domain robustly activates the DHFRGal4 reporter construct and that mutations which abolish mdm2 or Rb binding do not reduce activation. Therefore, recruitment of mdm2 or Rb is not required for E2F1-mediated activation of the *dhfr* promoter. This work is in agreement with the model that Rb functions to keep E2F1 inactive in G_0 phase cells but has no role in the S phase-mediated activation of promoters by E2F1.

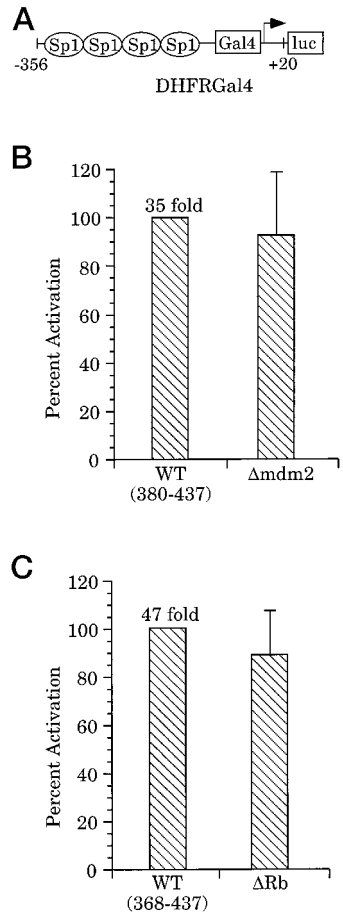


FIG. 2. **mdm2 and Rb binding are not critical for activation of the *dhfr* promoter.** *A*, schematic of the DHFRGal4 reporter construct containing a Gal4 binding site in precise replacement of the E2F1 binding site in the *dhfr* promoter. Activation of the DHFRGal4 reporter construct in NIH 3T3 cells by Gal4-E2F1 fusion proteins containing mutations that have been shown to abolish mdm2 binding (*B*) and Rb binding (*C*) was compared with the activation by the corresponding wild-type Gal4-E2F1 fusion proteins. Δmdm2 contains E2F1 amino acids 380–437 with D390A and F391A substitutions. ΔRb contains E2F1 amino acids 368–437 with a deletion of amino acids 418–422. The reporter construct (5 μg) and Gal4 fusion expression plasmids (5 μg) were transfected into 1.25×10^5 cells, which were then incubated in growth medium for 48 h, harvested, and analyzed for luciferase activity. -Fold activation was calculated by dividing the luciferase levels conferred to DHFRGal4 by the specified Gal4-E2F1 fusion protein by the luciferase levels conferred by the Gal4 DNA binding domain alone. The -fold activation by the wild-type E2F1 constructs (shown by the *number above* the *bar*) was set to 100%. Activation by the mutant Gal4-E2F1 fusion proteins is reported as a percentage of the activation conferred by the corresponding wild-type fusion protein. *Bars* represent standard errors of the means.

Previous studies have mapped the E2F1 transactivation domain to amino acids 380–437 or 399–437, using different minimal TATA box-containing synthetic promoter constructs with either one or three upstream Gal4 binding sites, respectively (38, 45). To determine the minimal region of E2F1 required for activation of *dhfr*, we employed a series of Gal4-E2F1 fusion proteins containing N-terminal and C-terminal deletions of the E2F1 transactivation domain (45). NIH 3T3 cells were cotransfected with the DHFRGal4 reporter construct and the different Gal4-E2F1 expression constructs. A Gal4-E2F1 fusion protein containing E2F1 amino acids 368–437 shows a 57-fold activation of the DHFRGal4 reporter construct, and deletion of N-terminal amino acids to position 399 does not

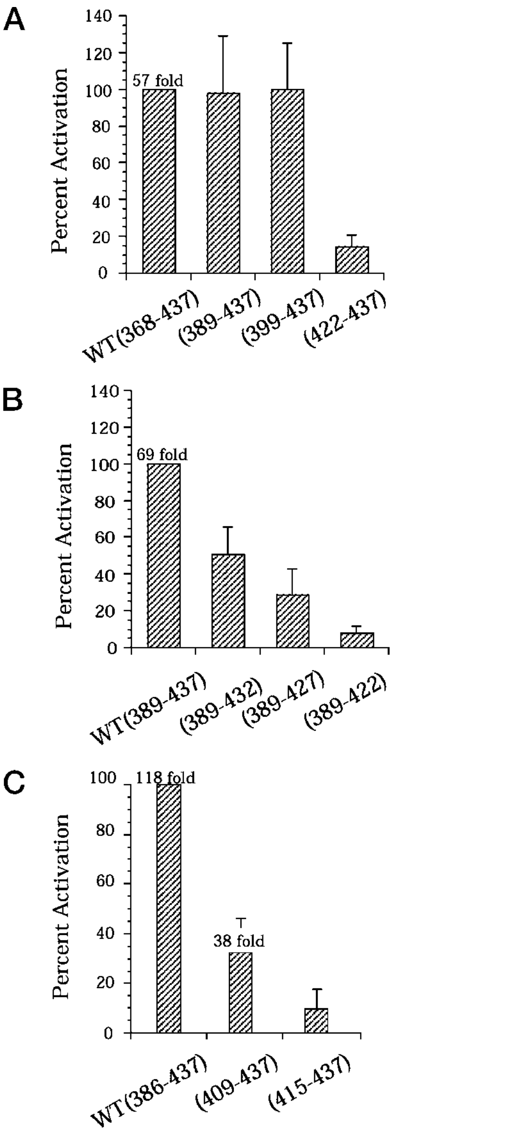


FIG. 3. **Amino acids 409–437 of E2F1 contain the core domain required for activation of the *dhfr* promoter.** Activation of the DHFRGal4 reporter construct by the indicated Gal4-E2F1 fusion proteins was examined in transiently transfected NIH 3T3 cells, as described in the legend to Fig. 2. Fusion proteins containing N-terminal (*A* and *C*) and C-terminal (*B*) truncations of the E2F1 protein identify regions of the transactivation domain required for activation of the *dhfr* promoter. -Fold activation of DHFRGal4 by the wild-type Gal4-E2F1 fusion proteins is given *above* the *bar*, while activation by other Gal4-E2F1 fusion proteins is reported as a percentage of the wild-type activation. *Bars* represent standard errors of the means.

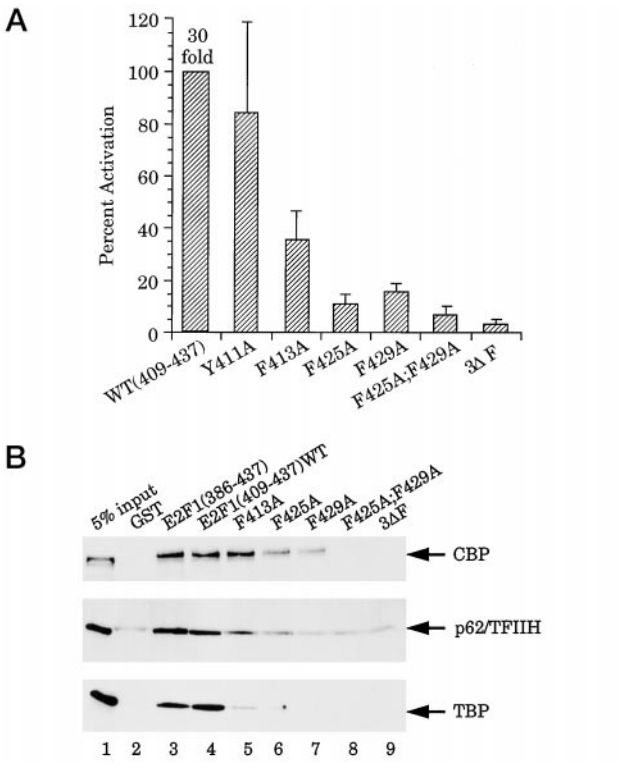
VP16(N)	L	D	G	E	D	V	A	M	A	H	A	D	A	L	D	D	F	D	L	D	M	L	G
VP16(C)	F	T	P	H	D	S	A	P	Y	G	A	L	D	M	A	D	F	E	F	EQM		F	T
Sp1(B)	I	R	T	P	T	V	G	P	N	G	Q	V	S	W	Q	T	L	Q	L	Q	N	L	Q
p53	S	V	E	P	P	L				S	Q	E	T	F	S	D	L		W	K	L	L	P
CREB	K	R	R	E	I	L			A	R	R	P	S	Y	R	R	I		L	D	D	L	A
E2F1	L		D	Y	H	F	G	L	E	E	G	E	G	I	RDL		F	D	C	D		F	G
E2F2	Q	D	D	Y	L	W	G	L	E	A	G	E	G	I	SDL		F	DS	Y	D		L	G
E2F3	Q	E	D	Y	L	L	S	L	G	E	E	E	G	I	SDL		F	DA	Y	D		L	E
E2F4	G	D	H	D	Y	I	YNL		D	E	S	E	G	V	CDL		F	D	V	P		V	L
E2F5	D	D	D	Y	N	F	N	L	D	D	N	E	G	V	CDL		F	D	V	Q		I	L

FIG. 4. **Bulky hydrophobic amino acids may be critical for E2F1 protein-protein interactions required for transcriptional activation.** Comparison of the primary sequences of various transactivation domains including E2F. This alignment is based on visual inspection of positionally conserved bulky hydrophobic amino acids (indicated in *boxes*) in E2F, p53, and CREB transactivation domains compared with the alignment of VP16 and Sp1, as reported by Cress and Triezenberg (54). Amino acid residues previously shown to be critical for protein-protein interactions and/or transactivation by VP16, Sp1, p53, and CREB are *underlined*. E2F1 amino acids 409–430 are depicted, and *underlined* residues were mutated in these studies.

reduce activation (Fig. 3*A*). This indicates that the region of E2F1 required for activation of *dhfr* does not extend as far N-terminal as the region previously shown to be required for activation of a minimal promoter containing one upstream Gal4 binding site (38). However, deletion of the next 23 N-terminal amino acids (399–422) does result in a 7-fold decrease in activation levels. An analysis of C-terminal deletions demonstrates that deletion of the last 5, 10, and 15 amino acids of E2F1 results in 2-, 4-, and 13-fold decreases in activation levels, respectively (Fig. 3*B*). Therefore, the extreme C terminus of E2F1 is critical for activation of *dhfr* transcription. Taken together, these results demonstrate that E2F1 amino acids 399–437 can confer robust activation to the *dhfr* promoter. To more precisely map the N-terminal boundary of the E2F1 transactivation domain, we utilized an additional series of N-terminal deletion constructs (39). We found that a Gal4-E2F1 fusion protein containing E2F1 amino acids 386–437 activated the DHFRGal4 reporter construct 118-fold, and a Gal4-E2F1 containing amino acids 409–437 retained 38-fold activation (Fig. 3*C*). However, further N-terminal deletion to amino acid 415 reduced activation to a very low level. Therefore, we define amino acids 409–437 as the core transactivation domain of E2F1.

Phenylalanine Residues in the E2F1 Core Transactivation Domain Are Critical for Activation of the dhfr Promoter and E2F1 Protein-Protein Interactions—Our deletion analysis of E2F1 identified a core domain that spans amino acids 409–437 and potently activates the *dhfr* promoter. Previous studies demonstrated that this domain binds to TBP and TFIIH (39); we show below that this domain is also sufficient to bind CBP (Fig. 5*B*). We next wanted to determine the contributions of TBP, TFIIH, and CBP binding to the activation of *dhfr* transcription by E2F1. A larger E2F1 transactivation domain is required for maximal activation of the *dhfr* promoter, but is also likely to bind additional unidentified proteins. Therefore, to simplify our analysis, we chose to study activation of *dhfr* transcription mediated by the core E2F1 transactivation domain. Further N- or C-terminal deletion of this 38-amino acid core region of E2F1 affects the binding of multiple target proteins (39, 53); therefore, a correlation of specific protein-protein interactions with transcriptional activation of *dhfr* required a more precise mutational analysis. Others have demonstrated that bulky hydrophobic amino acids in the VP16, p53, CREB, and Sp1 transactivation domains are critical for protein binding and transcriptional activation (6, 54–60). Based on amino acid sequence comparison of these transactivation domains (Fig. 4), we predicted that phenylalanine residues 413, 425, and 429 in the core E2F1 transactivation domain (*underlined* in Figs. 1 and 4) may be critical for protein binding and transcriptional activation. In support of this prediction, the transactivation domains of E2F2–5 also contain bulky hydrophobic residues at these aligned positions.

To identify the specific protein-protein interactions required for E2F1-mediated transcriptional activation of the *dhfr* promoter, we replaced phenylalanine residues 413, 425, and 429 in the core E2F1 transactivation domain with alanine. The effects of these alanine substitutions on transcriptional activation were determined by cotransfection of NIH 3T3 cells with the DHFRGal4 reporter construct and Gal4-E2F1 expression constructs. As shown in Fig. 5*A*, a wild-type E2F1 core transactivation domain shows a 30-fold activation of the DHFRGal4 reporter construct. Alanine substitution for Tyr-411, an amino acid residue that is highly conserved in E2F transactivation



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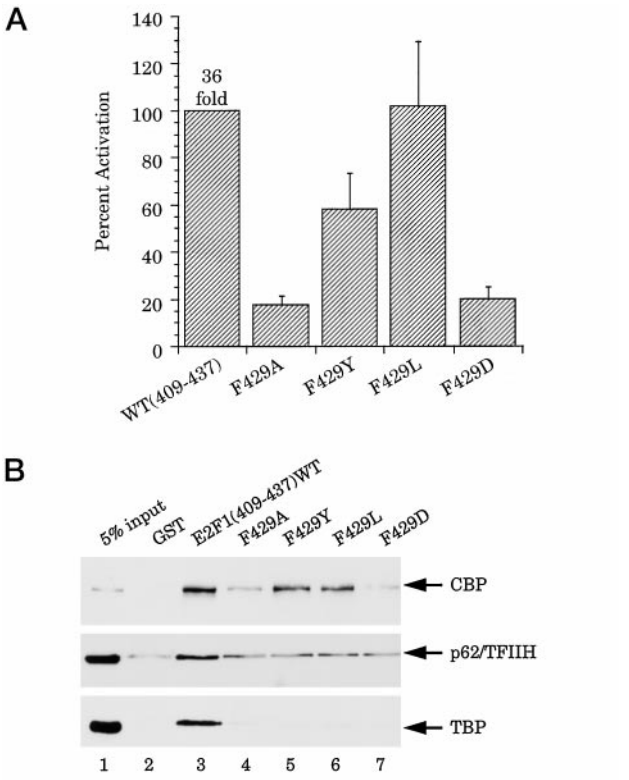
Construct	% Protein Binding CBP	TFIIH	TBP
E2F1 (386-437)WT	110	140	60
E2F1 (409-437)WT	100	100	100
E2F1 F413A	100	35	5
E2F1 F425A	40	–	–
E2F1 F429A	20	–	–
E2F1 F425A;F429A	–	–	–
E2F1 3ΔF	–	–	–

FIG. 5. **Phenylalanine residues in the core E2F1 transactivation domain are critical for both transcriptional activation and protein binding.** *A*, activation of the DHFRGal4 reporter construct by Gal4 fusion proteins containing the core E2F1 transactivation domain with the indicated alanine substitutions was examined in transiently transfected NIH 3T3 cells, as described in the legend to Fig. 2. -Fold activation of DHFRGal4 by the wild-type Gal4-E2F1 fusion protein is given *above* the *bar*, while activation by other Gal4-E2F1 fusion proteins is reported as a percentage of the wild-type activation. *Bars* represent standard errors of the means. *B*, HeLa nuclear extract was chromatographed over the indicated series of GST-E2F1 affinity columns. The columns were washed with low salt buffer and then eluted with buffer containing 1% SDS. The eluates were analyzed by Western blot analysis with antibodies directed against CBP, the p62 subunit of TFIIH, and TBP. The columns contained either GST alone (*lane 2*), GST-E2F1(386–437) (*lane 3*), or GST-E2F1(409–437) with the indicated amino acid substitutions (*lanes 4–9*). *Lane 1* contains 5% of the input HeLa nuclear extract. *Lanes 2–9* contain 40% (p62 and TBP) and 8% (CBP) of the indicated SDS eluates. Each binding experiment was performed at least four separate times. *C*, Western blot autoradiograms were scanned and the signals were quantitated using ImageQuant version 4.2a (Molecular Dynamics) to determine the relative levels of protein binding. The amount of each protein bound by the wild-type core E2F1 transactivation domain (amino acids 409–437) was set to 100%. Levels of protein bound by E2F1(386–437) and the mutant E2F1 transactivation domains is reported as a percentage of the protein bound by the wild-type core transactivation domain.

domains, but not positionally conserved in other transactivation domains, does not reduce activation of DHFRGal4. However, alanine substitution for Phe-413 reduces activation to 36% of wild-type levels, and substitutions for Phe-425 and Phe-429 reduce activation to 12% and 16% of wild-type levels, respectively. Double and triple alanine substitutions for residues Phe-413, Phe-425, and Phe-429 completely abolish activation, demonstrating that these residues are crucial for the activity of the core E2F1 transactivation domain. By Western blot analysis, we found the levels of wild-type and mutant Gal4-E2F1 fusion proteins in transfected NIH 3T3 cells to be equivalent (data not shown), demonstrating that the decreased activation observed for the mutant proteins is not a result of decreased expression.

To determine the effects of the alanine substitutions on protein binding, we compared CBP, TFIIH, and TBP binding by the wild-type and mutant E2F1 core transactivation domains. The smallest region of E2F1 previously shown to bind CBP, TFIIH, and TBP contained amino acids 380–437 (39, 53); therefore, we used a construct containing amino acids 386–437 as a positive control. HeLa nuclear extract was applied to protein affinity columns containing immobilized GST or GST-E2F1 fusion proteins. Bound proteins were eluted, subjected to SDS-PAGE, and analyzed by Western blot analysis using antibodies directed against CBP, the p62 subunit of TFIIH, and TBP. As expected, an E2F1 transactivation domain containing amino acids 386–437 binds to all three proteins (Fig. 5*B*, *lane 3*). We found that the core transactivation domain containing amino acids 409–437 is also sufficient for robust binding of CBP, TFIIH, and TBP (Fig. 4*B*, *lane 4*). Importantly, the phenylalanine substitutions that reduced activation had dramatic effects on protein binding (Fig. 5*B*, *lanes 5–9*; quantitated in Fig. 5*C*). TBP binding does not correlate with the ability of the E2F1 core transactivation domain to activate the *dhfr* promoter since alanine substitutions for Phe-413, Phe-425, and Phe-429 completely abolish TBP binding, but differentially reduce activation of DHFRGal4. However, binding of CBP and TFIIH more closely correlate with activation of DHFRGal4. Alanine substitutions for Phe-425 and Phe-429, which have the most dramatic effects on transcriptional activation, greatly reduce CBP binding and abolish TFIIH binding. Double and triple alanine substitutions for residues Phe-413, Phe-425, and Phe-429, which abolish activation of DHFRGal4, completely abolish binding to all three target proteins analyzed. By Coomassie-stained SDS-PAGE analysis, we found the amount of GST fusion proteins on the affinity columns to be equivalent (data not shown), demonstrating that the observed changes in TBP, TFIIH, and CBP binding are not due to differences in the amount of GST fusion protein.

Conservative Amino Acid Substitutions for Phenylalanine 429 Show a Strong Correlation between Recruitment of CBP and Activation of the dhfr Promoter by E2F1—Our initial mutational analysis of the core E2F1 transactivation domain demonstrated the importance of phenylalanine residues 413, 425, and 429 for both transcriptional activity of E2F1 and binding of CBP, TFIIH, and TBP. Unfortunately, we were unable to identify which of these target proteins is required for activation of the *dhfr* promoter, since alanine substitutions for these residues reduced binding to all three proteins. However, we postulated that if loss of protein binding due to replacement of the phenylalanine residues with alanine was a result of loss of a critical bulky hydrophobic side group, then conservative replacement with residues containing other bulky hydrophobic side groups may restore transcriptional activation, while restoring only a subset of protein-protein interactions. Since alanine substitution for Phe-429 had the most dramatic effect on binding of all three proteins tested, we replaced this residue with tyrosine and leucine to determine if other aromatic and/or bulky hydrophobic residues could function in place of phenylalanine in activation and protein binding. We also replaced Phe-429 with the charged residue aspartate to further test the requirement for a hydrophobic residue at this position. To determine the effects of these substitutions on transcriptional



C

Construct	% Protein Binding CBP	TFIIH	TBP
E2F1 (409-437)WT	100	100	100
E2F1 F429A	15	30	-
E2F1 F429Y	85	25	-
E2F1 F429L	70	30	-
E2F1 F429D	10	15	-

FIG. 6. **CBP binding correlates with activation of the *dhfr* promoter by the core E2F1 transactivation domain.** *A*, activation of the DHFRGal4 reporter construct by Gal4-E2F1 fusion proteins containing the indicated Phe-429 substitutions was examined in transiently transfected NIH 3T3 cells, as described in the legend to Fig. 2. -Fold activation of DHFRGal4 by the wild-type Gal4-E2F1 fusion protein is given *above* the *bar*, while activation by the mutated Gal4-E2F1 fusion proteins is reported as a percentage of the wild-type activation. *Bars* represent standard errors of the means. *B*, CBP, TFIIH, and TBP binding by core E2F1 transactivation domains containing the indicated Phe-429 substitutions was measured as described in the legend to Fig. 4. The columns contained either GST alone (*lane 2*), GST-E2F1(409–437) (*lane 3*), or GST-E2F1(409–437) with the indicated Phe-429 substitutions (*lanes 4–7*). *Lane 1* contains 5% of the input HeLa nuclear extract. *Lanes 2–7* contain 40% (p62 and TBP) and 8% (CBP) of the indicated SDS eluates. Each binding experiment was performed at least four separate times. *C*, protein binding by the wild-type and Phe-429 substituted core E2F1 transactivation domains was quantitated as described in the legend to Fig. 4. The amount of each protein bound by the wild-type core E2F1 transactivation domain was set to 100%. Levels of protein bound by Phe-429 substituted E2F1 transactivation domains is reported as a percent of the protein bound by the wild-type core transactivation domain.

activation, we cotransfected NIH 3T3 cells with the DHFRGal4 reporter construct and Gal4-E2F1 expression constructs. As expected, the wild-type E2F1 core transactivation domain shows a 36-fold activation of the DHFRGal4 reporter construct, and the alanine substitution for Phe-429 reduces activation to 17% of wild-type levels (Fig. 6*A*). However, replacement of Phe-429 with tyrosine or leucine either partially or completely restores activation to 58% and 102% wild-type levels, respectively. An aspartate residue at position 429 reduces activation to 20% wild-type levels. The difference in levels of activation observed for the mutant proteins is not a result of differential expression, since by Western blot analysis, we found the levels of wild-type and mutant Gal4-E2F1 fusion proteins in transfected NIH 3T3 cells to be equivalent (data not shown). Taken together, our results demonstrate that tyrosine and leucine can function in place of Phe-429 of the core E2F1 transactivation domain in the activation of the *dhfr* promoter. It is also interesting to note that the transactivation domains of E2F2 and E2F3 contain a leucine residue at this position (see Fig. 4).

To determine if replacement of phenylalanine 429 with tyrosine, leucine, or aspartate altered the binding of target proteins to the core E2F1 transactivation domain, we compared CBP, TFIIH, and TBP binding by the wild-type and mutant transactivation domains. HeLa nuclear extract was applied to protein affinity columns as described above. Bound proteins were eluted and analyzed by Western blot analysis using antibodies directed against CBP, the p62 subunit of TFIIH, and TBP. We found that tyrosine and leucine substitutions for Phe-429 do alter target protein binding by the core E2F1 transactivation domain. As shown in Fig. 6*B* (quantitated in Fig. 6*C*), replacement of Phe-429 with tyrosine, leucine, or aspartate greatly reduces TFIIH and TBP binding to levels indistinguishable from that of F429A (compare *lanes 5–7* to *lane 4*). However, only replacement of Phe-429 with tyrosine and leucine, both of which can substitute for Phe-429 in activation of DHFRGal4, result in appreciable binding of CBP when compared with wild-type (Fig. 6*B*, compare *lanes 4–7* to *lane 3*). F429A and F429D, which do not activate DHFRGal4, bind only very low amounts of CBP. By Coomassie-stained SDS-PAGE analysis, we found the amount of GST fusion proteins on the affinity columns to be equivalent (data not shown), demonstrating that the observed changes in TBP, TFIIH, and CBP binding are not due to differences in the amount of GST fusion protein. Taken together, our results show a strong correlation between activation of DHFRGal4 and binding of the core E2F1 transactivation domain to CBP, but not TFIIH or TBP. Therefore, we suggest that CBP recruitment is critical for activation of the *dhfr* promoter.

Transactivator Bypass Experiments Indicate That Recruitment of CBP Is Sufficient for Activation of the dhfr Promoter—We have defined a core E2F1 transactivation domain containing only 38 amino acids and have shown a strong correlation between activation of the *dhfr* promoter and binding of CBP, but not TFIIH or TBP. We next wished to determine if the direct recruitment of either CBP, TFIIH, or TBP could bypass the requirement for the E2F1 transactivation domain at the *dhfr* promoter. Therefore, we analyzed the ability of Gal4-CBP, Gal4-p62 (TFIIH), and Gal4-TBP fusion proteins to activate the DHFRGal4 reporter construct in NIH 3T3 cells (11, 61). Since TFIIH is a multisubunit protein complex, we chose to fuse the Gal4 DNA binding domain to the p62 subunit which has previously been shown to directly contact the E2F1 transactivation domain (39). As shown in Fig. 7, Gal4-p62 and Gal4-TBP show only a 2.1- and 1.2-fold activation of DHFRGal4, respectively. The inability of these fusion proteins to activate transcription in a transactivator bypass experiment suggests that recruitment of TFIIH and TBP are not rate-limiting steps in the activation of the *dhfr* promoter. However, a Gal4-CBP fusion protein shows an 8.5-fold activation of DHFRGal4, suggesting that recruitment of CBP is rate-limiting in the activation of *dhfr*. Therefore, we suggest that recruitment of CBP is one mechanism by which E2F1 activates *dhfr* transcription.

DISCUSSION

We have previously demonstrated that the murine *dhfr* promoter is activated by an E2F family member in S phase (29). We have also shown that E2F1 can bind to the *dhfr* promoter *in vivo*[2] and that E2F1 is a robust activator of *dhfr* transcription

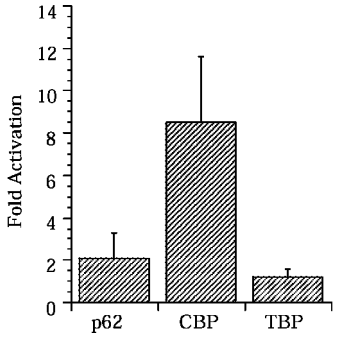


FIG. 7. **CBP activates the *dhfr* promoter in an E2F1 transactivator bypass experiment.** Activation of the DHFRGal4 reporter construct by Gal4 fusion proteins containing the p62 subunit of TFIIH, CBP, and TBP was examined in transiently transfected NIH 3T3 cells, as described in the legend to Fig. 2. -Fold activation was calculated by dividing the luciferase levels conferred to DHFRGal4 by the specified Gal4 fusion protein by the luciferase levels conferred by the Gal4 DNA binding domain alone. *Bars* represent standard errors of the means.

(29, 62). Therefore, we have chosen E2F1 as the representative E2F family member in our experiments designed to define and characterize the region of E2F proteins and protein-protein interactions required for activation of *dhfr* transcription. The E2F1 transactivation domain binds a variety of cellular proteins including mdm2, Rb, CBP, TFIIH, and TBP (17, 38, 39, 46, 53). Many of these protein-protein interactions have been shown to correlate with activation of synthetic TATA-containing promoters (38, 39, 46, 53); however, we found only CBP binding to correlate with activation of the *dhfr* promoter. Using deletion analysis, we have shown a region of E2F1 containing amino acids 399–437 to be sufficient for maximal activation of the *dhfr* promoter, and identified a smaller core domain of E2F1 spanning amino acids 409–437 that retains robust activation and binding of CBP, TFIIH, and TBP. Further analysis using amino acid substitutions indicated that two phenylalanine residues in the core domain (Phe-425 and Phe-429) are highly critical for both transcriptional activation of *dhfr* and binding of CBP, TFIIH, and TBP. However, further amino acid substitutions for Phe-429 eliminated the correlation between activation and binding of TFIIH and TBP. In contrast, our results showed a striking correlation between the ability of the core E2F1 transactivation domain to activate the *dhfr* promoter and to bind CBP. Furthermore, in a transactivator bypass experiment, we demonstrated that direct recruitment of CBP results in activation of the *dhfr* promoter. Therefore, we suggest that recruitment of CBP is one mechanism by which E2F1 activates the *dhfr* promoter.

Our results strongly suggest that recruitment of mdm2, Rb, TFIIH, and TBP are not required for activation of the *dhfr* promoter by E2F1. For example, Gal4-E2F1 fusion proteins that no longer bind to mdm2 or Rb retain the ability to activate the *dhfr* promoter (Fig. 2). Additionally, Gal4-E2F1 fusion proteins that no longer bind to TFIIH or TBP retain the ability to activate the *dhfr* promoter (Figs. 5 and 6), and neither Gal4-TBP nor Gal4-p62 activated the *dhfr* promoter in a transactivator bypass experiment, suggesting that recruitment of TFIIH and TBP are not rate-limiting steps in activation of *dhfr* transcription. It is not surprising that E2F1 is not required to recruit TBP, since the *dhfr* promoter contains four binding sites for Sp1, which has been shown to bind other subunits of TFIID and to substitute for a TATA box as the primary transcription initiation positioning element (6, 63–66). Our results are also in complete agreement with previous studies demonstrating that recruitment of TBP, a component of the general transcription factor TFIID, is not rate-limiting for TATA-less promoters (22). In addition, we have found that several transactivation domains which bind TBP, including a C-terminally truncated VP16 transactivation domain, are not able to activate *dhfr* transcription (29).

Although our studies suggest that CBP recruitment is one essential function of E2F1, we also note that additional proteins may be required for maximal activation of *dhfr* transcription. For example, the core transactivation domain does not activate the *dhfr* promoter to the same level as does a longer E2F1 transactivation domain, even though both constructs bind similar amounts of CBP (Fig. 5). In addition, alanine replacement of Phe-413 decreased activation by 3-fold, but had no apparent effect on CBP binding by the core E2F1 transactivation domain. These results could be due to the *in vitro* conditions chosen to examine binding of E2F1 to CBP or could be due to a requirement for other unidentified activities. Regardless, our results strongly suggest that recruitment of CBP is critical for activation of the *dhfr* promoter.

CBP is a member of a family of global transcriptional coactivators that is involved in the regulation of many DNA binding transcriptional activators (for recent reviews, see Refs. 67–69). Previous studies suggest several mechanisms by which CBP may activate transcription. For example, CBP can directly interact with RNA polymerase II, TFIIB, and TBP, suggesting a role in recruitment of the basal transcription machinery (70, 71). In addition, CBP has been shown to contain histone acetyltransferase activity and to associate with proteins that also have similar activities, including p300/CBP-associated factor (72, 73). Acetylation of the N-terminal tails of histones has long been correlated with transcriptional activation (for a recent review, see Ref. 74), and our preliminary data suggests that the *dhfr* promoter may be regulated by changes in histone acetylation. First, we find that the histone deacetylase inhibitor trichostatin A induces transcription from the endogenous *dhfr* promoter in quiescent NIH 3T3 cells.[3] Furthermore, using a chromatin immunoprecipitation assay with antibodies directed against acetylated histones, we find an increase in the abundance of acetylated histone H3 at the endogenous *dhfr* promoter in mid to late G_1 phase of the cell cycle, just prior to activation of transcription.[2] These experiments indicate that a transition from deacetylated to acetylated histones correlates with activation of the *dhfr* promoter. Therefore, we propose that E2F1-mediated activation of the *dhfr* promoter involves the histone acetylation activity of CBP. However, CBP has also been shown to acetylate other proteins, including the general transcription factors TFIIEβ and TFIIF (75). Therefore, it remains possible that E2F1 recruitment of CBP may be to achieve acetylation of another component of the transcriptional machinery.

In summary, we propose a model (shown in Fig. 8) in which transcription of the *dhfr* promoter is repressed in G_0 phase due to the association of E2F with a pocket protein (such as Rb), which masks the E2F transactivation domain, blocks recruitment of CBP, and recruits histone deacetylase (HDAC) activity. In support of this model, it has been shown that Rb, p107, and p130 all share the ability to repress E2F activity through recruitment of HDAC1 (35, 37, 76). Upon phosphorylation and release of the pocket proteins in late G_1 phase of the cell cycle, E2F recruits CBP, which acetylates histone H3. The structural changes in the nucleosome that result as a consequence of acetylation of H3 then contribute to the activation of *dhfr* transcription, possibly by increasing the access of RNA polymerase and the basal machinery to the promoter. Although our studies have been performed using transiently transfected plasmids, we feel that our results are likely to be relevant to the endogenous *dhfr* promoter. First, we have shown that cell cycle regulation is maintained with transiently transfected *dhfr* re-

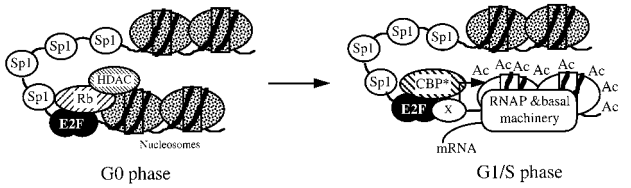


FIG. 8. **A model for E2F1-mediated activation of the *dhfr* promoter in S phase.** Figure is a schematic depicting the murine *dhfr* promoter in G_0 and G_1/S phase of the cell cycle; the *arrow* represents the transcription start site. Transcription from the *dhfr* promoter is low in G_0 phase because: 1) Rb binds to and masks the E2F transactivation domain and 2) Rb recruits HDAC1 which deacetylates histones, maintaining a chromatin structure that hinders access of RNA polymerase II and the basal transcription machinery. Rb is phosphorylated and released from E2F in late G_1 and early S phase, allowing for recruitment of CBP and associated factors (CBP*). CBP* then acetylates histones, resulting in a change of chromatin structure (depicted by differential *shading* of nucleosomes) and stimulation of *dhfr* transcription. We note that E2F may also recruit additional unidentified proteins (represented by *protein X*) that are required for maximal activation of the *dhfr* promoter.

porter plasmid (28, 29). Second, previous studies have demonstrated that transfected plasmids interact with histones to form nucleosome-like structures and that recruitment of histone deacetylases or histone acetylases can modulate transcription from such plasmids (37, 77–81). However, we also realize that a more complete understanding of *dhfr* regulation may come from an analysis of a chromosomally located promoter. Therefore, studies are in progress to compare cell cycle stage-specific changes in acetylation using integrated and episomal *dhfr* promoter constructs.

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