Position-Dependent Transcriptional Regulation of the Murine Dihydrofolate Reductase Promoter by the E2F Transactivation Domain

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Activity of the dihydrofolate reductase (dhfr) promoter increases at the G1-S-phase boundary of the cell cycle. Mutations that abolish protein binding to an E2F element in the dhfr promoter also abolish the G1-S-phase increase in dhfr transcription, indicating that transcriptional regulation is mediated by the E2F family of proteins. To investigate the mechanism by which E2F regulates dhfr transcription, we moved the E2F element upstream and downstream of its natural position in the promoter. We found that the E2F element confers growth regulation to the dhfr promoter only when it is proximal to the transcription start site. Using a heterologous E2F element, we showed that position-dependent regulation is a property that is promoter specific, not E2F element specific. We demonstrated that E2F-mediated growth regulation of dhfr transcription requires activation of the dhfr promoter in S phase and that the C-terminal activation domains of E2F1, E2F4, and E2F5, when fused to the Gal4 DNA binding domain, are sufficient to specify position-dependent activation. To further investigate the role of activation in dhfr regulation, we tested other transactivation domains for their ability to activate the dhfr promoter. We found that the N-terminal transactivation domain of VP16 cannot activate the dhfr promoter. We propose that, unlike other E2F-regulated promoters, robust transcription from the dhfr promoter requires an E2F transactivation domain close to the transcription start site.

The E2F family of heterodimeric transcription factors plays an important role in regulating gene expression at the G1-S-phase transition of the mammalian cell cycle. Seven members of this family have been identified: five different E2F proteins (E2F1 to E2F5) and two different DP proteins (DP1 and DP2). E2F and DP proteins bind to DNA as a heterodimer to form a functional E2F complex that contributes to the regulation of many promoters (for recent reviews, see references 10 and 51). Cellular promoters regulated by E2F include genes required for DNA synthesis (dihydrofolate reductase [dhfr], thymidine kinase, and DNA polymerase α) and transcriptional regulators of cell growth (N-myc, c-myc, B-myb, cdc2, E2F1, retinoblastoma [Rb], p107, and cyclins E and A) (5, 20, 44, 48, 51, 60).

E2F is believed to confer growth regulation to the E2F1, B-myb, and cdc2 promoters by mediating transcriptional repression in the G0 phase of the cell cycle (11, 23, 25, 33, 34, 42, 61). For example, mutation of the E2F element in the murine B-myb promoter results in constitutively high levels of transcription in mouse fibroblasts and human keratinocytes (33, 34). Also, in vivo footprinting analysis of the B-myb promoter shows occupation of the E2F element only in G0-phase cells when the B-myb promoter is inactive (61). This transcriptional repressor activity of E2F is attributed to its interaction with the Rb family of 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 the p107 and p130 proteins (for a recent review, see reference 8). Transcriptional activation by E2F can be masked by pocket proteins, since they bind to a region which overlaps the transactivation domain of E2F (for reviews, see references 32 and 43). In addition, Rb has been shown to repress transcription mediated by other transcriptional activator proteins when recruited to a promoter by the Gal4 DNA binding domain (56, 57). E2F-pocket protein complexes are most abundant in G0 and early G1 phase. In late G1 and early S phase, the pocket protein is phosphorylated by cyclin D- and cyclin E-dependent kinases and subsequently released from E2F-DP complexes. Thus, it is proposed that E2F mediates differential levels of transcription in G0 versus S phase by recruiting an Rb family member which represses transcription in G0 phase.

However, previous studies suggest that E2F-mediated repression cannot account for the growth regulation of the dhfr promoter. We have shown that mutation of the dhfr E2F element results in only a two- to threefold increase in promoter activity in quiescent cells (50). Also, unlike the B-myb promoter, 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 G0 and early S phase; the other strand is constitutively occupied (58). We have further investigated the possibility that dhfr may be regulated differently than other E2F element-containing promoters, and we now present evidence that growth regulation of the murine dhfr promoter is achieved by position-dependent E2F-mediated activation of transcription in S phase.

MATERIALS AND METHODS

Plasmids. Standard cloning techniques were used for all plasmid constructions (38). All dhfr reporter plasmids contain promoter fragments cloned upstream of the luciferase cDNA in the vector pAAlucA (21). The pMaeWT and pMaeE2F reporter constructs contain dhfr sequences from positions −270 to +20 and have been described previously as pWTluc and pNWluc, respectively (41). pMaeE2F@−375 was created by inserting a double-stranded oligonucleotide containing the wild-type dhfr promoter sequence from −20 to +9 plus a PvuII site for screening and SacII sites at both ends into SacII-digested pMaeE2F. The sequence of the top strand of the oligonucleotide reads 5′-CA
GCTGCGTCGATTTCCGGCCTACTGACGGCCG-3', with the E2F element indicated in boldface type. The E2F element was placed in the same orientation as in the wild-type dhp promoter. The same cloning scheme was used to create pMaAcE2F@-375, which contains two copies of the oligonucleotide in direct orientation, and pMaAcE2F@-375, which contains one copy of this oligonucleotide in the orientation opposite than in pMaAcE2F@-375. The same cloning scheme was also used to create pMaAc-bmyb@-375 and pMaBmyb-B@-375 by inserting a double-stranded oligonucleotide (top strand reads 5'-GAGGCCAGAGACTCTCCGATCCGCA- CAGCTGCGTCGATTTCCGGCCTACTGACGGCCG-3') into the cloning site ATGCCTGGCAATCTAGACTAGTCTAGACGC-3'), which contains the PstI site for cloning and EcoRI and XmaI sites at the 5' and 3' ends, respectively, was placed into the EcoRI- and XmaI-digested pBSM13 plasmid containing the dhp fragment. Finally, the plBSM13 plasmid containing the dhp fragment and PstI linker was digested with PstI, and the fragment containing the dhp sequence was placed into PstI-digested pAluAaC. The resulting pl410WT reporter construct contains dhp promoter sequences from -356 to +61.

The pl410WT plasmid was digested with SacI and the PstI linker used to clone pl410WT was placed into EcoRI- and XmaI-digested pSTU+14mp19 plasmid (40). The pSTU+14mp19 plasmid containing the PstI linker was then digested with PstI, and the fragment containing the dhp fragment was placed into PstI-digested pAluAaC. The new 410WT reporter construct is identical to pl410WT except it contains a 14bp linker oligonucleotide (top strand reads 5'-CTAGTCTAGACTAGTCTAGACGC-3'), inserted into the Stnl site, p410E2F@+2 and p410E2F@+6 were created by inserting two copies of the 21bp oligonucleotide 5'-CTAGTCTAGACTAGTCTAGACGC-3') and two copies of the 33-bp oligonucleotide 5'-CTAGTCTAGACTAGTCTAGACGC-3') into Stnl-digested p410WT.

To create p410Myb-M-p410WT was digested with SmlI and HindIII to remove the 61 region of the dhp promoter and the 5' flanking region was then replaced by a double-stranded oligonucleotide (top strand reads 5'-CCTCGGCAATCTAGACGC-3') which contains Stnl and HindIII sites at the 5' and 3' ends and the dhp promoter sequence from positions -17 to +20, but the dhp linker element is replaced precisely with the E2F element from the B-myb promoter (indicated in boldface type). This construct contains the B-myb E2F element in the orientation opposite that in the B-myb promoter.

DHFRGal4 was created by replacing the -17 to +61 region of the dhp promoter and the 5' flanking region with a mutated dhp fragment containing a mutated E2F element. The new 410WT reporter construct contains five Gal4 elements upstream of the adenosine virus major late promoter TATA box and murine terminal deoxynucleotidyltransferase initiator element (Inr) cloned upstream of the luciferase cDNA in the vector pGL2Basic (Promega).

DHFRGal4@-375 was created by inserting a double-stranded oligonucleotide (top strand reads 5'-GGAGGCCAGAGACTCTCCGATCCGCA- CAGCTGCGTCGATTTCCGGCCTACTGACGGCCG-3') into SacI-digested pMaAcE2F. This oligonucleotide contains a Gal4 element (indicated in boldface type), a SmlI restriction site for screening, and SacI sites at both ends. Gal4-E2F1(368-437), Gal4-E2F2(276-412), and Gal4-E2F3(225-346) all contain human E2F sequence and have been described previously (17, 22, 26). The Rb-binding-deficient Gal4-E2F1 contains human E2F1 sequence and has been described previously as Gal4-E2F1(368-437) (d423-427) (17). The Gal4 fusion to the N terminus of E2F1 has been described previously as Gal4-E2F1(1-163) (17). Gal4-VPl6 (49) and Gal4(1-147) (17) have been described previously.

Cell culture and transfection. NIH 3T3 cell cultures were maintained as described previously (41). Calcium phosphate transfections were performed as described previously (41) with the following alterations. One day prior to transfection, 1.0 x 10^5 (growing cell experiments) to 1.3 x 10^5 (serum starvation and stimulation experiments) cells were seeded into 60-mm diameter dishes. For serum starvation and stimulation experiments, each dish of cells was trypsinized with 5 μg of trypsin and 10 μg of DNase I and 10 μg of sonicated salmon sperm DNA. For growing cell experiments, each dish of cells was transfected with 5 μg of reporter DNA, 5 μg of Gal4 fusion expression DNA, and 5 μg of sonicated salmon sperm DNA. Each dish received a total of 15 μg of DNA as a precipitate in 450 μl of transfection buffer (41) and 50 μl of 1.0 M CaCl_2. At 6 h after transfection, cells were washed once with DMEM (Dulbecco’s modified Eagle’s medium [DMEM]) (GIBCO-BRL)-100 U of penicillin per ml-100 μg of streptomycin [GIBCO] per ml), shocked for 4 min with 1 ml of 15% (vol/vol) glycerol in transfection buffer, rinsed twice with rinse medium, and incubated in the appropriate medium. For serum starvation and stimulation experiments, the cells were induced to growth arrest by immediate incubation in starvation medium (DMEM plus 0.5% [vol/vol] bovine calf serum [HyClone]) for 48 to 60 h. The cells were then stimulated to reenter the proliferative cell cycle by replacing the starvation medium with simultaneous serum (DMEM) (GIBCO-BRL)-100 U of penicillin per ml-100 μg of streptomycin [GIBCO] per ml) and harvested at 0 h (starved cells) and 12 h (S-phase cells with peak levels of dhp transcription) after serum stimulation, and total cell lysates were assayed for luciferase activity. For growing cell experiments, the cells were incubated in growth medium (DMEM plus 5% [vol/vol] bovine calf serum) for 40 to 48 h before harvesting, and total cell lysates were assayed for luciferase activity. Each transfection was repeated at least two times with duplicate samples and multiple DNA preparations.

For luciferase assays, cells were rinsed once with phosphate-buffered saline, scraped from the plates in phosphate-buffered saline containing 1 mM EDTA, and pelleted for 4 min at 4°C in a microcentrifuge at 14,000 rpm. Cell pellets were resuspended in 100 μl of luciferase lysis buffer (Promega), lysed on ice for 7 min, and spun at 10,000 rpm in a microfuge for 10 min. Cell lysates were assayed for luciferase activity. For growing cell experiments, the cells were incubated in growth medium (DMEM plus 5% [vol/vol] bovine calf serum) for 40 to 48 h before harvesting, and total cell lysates were assayed for luciferase activity. Each transfection was repeated at least two times with duplicate samples and multiple DNA preparations.

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induced to enter quiescence by using serum starvation medium. The starvation medium was then replaced with serum stimulation medium, causing the cells to enter the proliferative cycle as a synchronous population. Cells were harvested and assayed for luciferase activity at 0 h (quiescent cells) and 12 h (S-phase cells) after serum stimulation, and the increase in activity of each reporter construct after serum stimulation is shown as fold S-phase induction (Fig. 1B). The activity of pMaeWT, which contains a functional E2F element, increased approximately 14-fold after serum stimulation, while the activity of pMaeΔE2F, which contains a mutated E2F element, increased only 4-fold (Fig. 1B). This residual activation of pMaeΔE2F is conferred by the four Sp1 elements positioned upstream of the E2F element (47). The activity of the reporter constructs with one or two copies of the E2F element positioned at nucleotide −375 increased only three- to fourfold (Fig. 1B). Thus, these constructs do not show the E2F-mediated increase in transcription levels after serum stimulation. This finding demonstrates that the E2F element cannot confer growth regulation from a distance upstream of the core dhfr promoter.

To determine if the E2F element could confer growth regulation from a position downstream of the core dhfr promoter, we moved the E2F element 14, 42, and 66 bp downstream of the wild-type position (Fig. 1C). NIH 3T3 cells were transiently transfected with the dhfr–luciferase reporter constructs, serum starved, and stimulated as described above for the upstream reporter constructs. The activity of the wild-type dhfr promoter increased approximately 12-fold after serum stimulation (Fig. 1D). As the E2F element was moved further downstream from...
the wild-type position, the S-phase induction of dhfr promoter activity gradually decreased (Fig. 1D). For example, the activity of the dhfr promoter construct having an E2F element at +66 increased only twofold after serum stimulation (Fig. 1D). Taken together, our results indicate that the E2F element cannot confer growth regulation to the dhfr promoter from a distance, either upstream or downstream of the wild-type position.

Although our results suggest that loss of growth regulation of the dhfr promoter is due to the movement of the E2F element, it was possible that loss of regulation was actually due to the movement of the site of transcription initiation. Based on the transcriptional activity of a dhfr promoter with the E2F element deleted, we previously proposed that protein binding to the E2F element positioned the start site of transcription (40). However, the E2F element overlaps a consensus initiator element (a sequence that spans the transcription start site of many promoters and may play a critical role in the positioning of transcription) located at nucleotides −2 to +6 in the dhfr promoter (28, 40, 46). Subsequent experiments using several different point mutations showed that mutation of the E2F element only alters the transcription start site when the mutation also alters the −1 to +2 region of the promoter (28, 46), suggesting that E2F is not critical for positioning of the transcription start site. Other studies have implicated Sp1 in the positioning of transcription initiation, demonstrating that an Sp1 element can substitute for a TATA box as the primary positioning element and direct transcription initiation approximately 50 bp downstream (2, 29). Therefore, it is likely that the Sp1 elements, in combination with the −1 to +2 region in the murine dhfr promoter direct efficient transcription at the initiation site (14, 19, 40, 53).

Although we expected the Sp1 elements to specify the position of the start site in our dhfr promoter-reporter constructs, we felt it necessary to demonstrate that the position of the transcription start site did not change when the E2F element was moved. Therefore, we analyzed three non-growth-regulated dhfr promoter constructs by using an in vitro transcription assay with HeLa cell nuclear extract. pMaeΔE2F and pMaeE2F@−375, both of which contain the wild-type −1 to +2 region of the dhfr promoter (CAA), produced RNA transcripts the same length as the transcript produced by pMaeWT (Fig. 2A). p410E2F@+66 produced an RNA transcript approximately 66 nucleotides longer than the RNA transcript produced by p410WT (Fig. 2B), indicating that even though the E2F element was moved 66 bp downstream, the approximate position of the transcription start site relative to the upstream Sp1 elements was maintained. These results indicate that the approximate position of the transcription start site relative to the core dhfr promoter region does not change when the E2F element is moved upstream or downstream of its wild-type position. Also, the transcriptional activity of pMaeE2F@−375 and p410E2F@+66 was similar to the activity of their wild-type counterparts, indicating that movement of the E2F element did not cause a decrease in levels of basal transcription in vitro.
from the displacement of the E2F element is not due to constructs. We find that loss of growth regulation versus non-growth-regulated 
represents standard errors of the means. Luciferase values are reported as relative luciferase units (RLu), which directly
represent standard errors of the means.

Our experiments indicate that although the E2F element is not involved in the positioning of the start site, the position of the E2F element in the dhfr promoter is critical for growth regulation. Others have shown that an E2F element can confer Rb-mediated transcriptional repression to the simian virus 40 (SV40) enhancer from a distance of 2 kb upstream, suggesting that repression is independent of the position of the E2F element (56). Therefore, our results suggest a different role for E2F in the regulation of the dhfr promoter. Support for the hypothesis that dhfr is not regulated by Gs-specific repression comes from a comparison of actual promoter activities of growth-regulated versus non-growth-regulated dhfr promoter constructs. We find that loss of growth regulation resulting from the displacement of the E2F element is not due to increased promoter activity in G0-phase cells (which would be expected if loss of repression occurred) but rather is due to decreased promoter activity in S-phase cells (Fig. 3). Taken together, our results indicate that the E2F element confers growth regulation to the dhfr promoter by mediating position-dependent activation of transcription in S phase.

The mechanism by which E2F mediates growth regulation is core promoter specific, not E2F element specific. Although our studies suggest that the E2F element is necessary for high levels of transcription from the dhfr promoter, E2F elements in other promoters, including B-myb, are thought to regulate activity through the cell cycle by conferring Gs-specific transcriptional repression (33, 34, 61). To determine if these distinct mechanisms of E2F-mediated growth regulation are specified by the E2F element or by the core promoter, we replaced the E2F element in the dhfr promoter with the E2F element from the murine B-myb promoter (Fig. 4A). Given that an E2F element can confer Rb-mediated repression from a distance of 2 kb upstream of a core promoter (56), the B-myb E2F element should be able to repress the dhfr promoter when positioned at nucleotide −375. Therefore, if Gs-phase repression is an intrinsic property of the E2F element, the B-myb “repressor” E2F element should confer growth regulation to the dhfr promoter from both the wild-type and upstream positions.

In contrast, if position-dependent S-phase-specific activation of transcription is specified by the core dhfr promoter, regulation of the dhfr promoter by the B-myb E2F element should be identical to regulation by the dhfr E2F element.

For a positive control, we first determined if the B-myb E2F element could confer growth regulation to the dhfr promoter if it is proximal to the transcription start site (Fig. 4B and C). Both the dhfr and B-myb E2F elements consist of two overlapping and inverted E2F consensus binding sites. Thus, the orientation of the B-myb element should not affect E2F activity. However, the sequences flanking either side of the B-myb E2F element are different. Therefore, we placed the B-myb E2F element in the orientation that creates a natural transcription start element (CAA from positions −1 to +2). NIH 3T3 cells were transfected with dhfr promoter constructs having the dhfr E2F element, B-myb E2F element, or no E2F element and then serum starved and stimulated as described above. The activity of p410WT, which contains the dhfr E2F element, increased 14-fold after serum stimulation, while the activity of p410AE2F, which contains a mutated E2F element, increased only 4-fold (Fig. 4C). The activity of p410bym-B, which contains the B-myb E2F element, increased approximately 10-fold after serum stimulation (Fig. 4C). Deviation of the B-myb E2F binding sites from the consensus B-myb binding site may account for the lower increase in promoter activity after serum stimulation. Thus, the B-myb E2F element can confer growth regulation to the dhfr promoter if placed proximal to the transcription start site.

To determine if the B-myb E2F element can confer growth regulation from a position upstream of the core dhfr promoter, we placed the B-myb E2F element in both orientations 375 bp upstream of the transcriptional start site of a core dhfr promoter containing a mutated E2F element in the wild-type position (Fig. 4D). NIH 3T3 cells were transfected with the dhfr-luciferase reporter constructs, serum starved, and stimulated as described above. The activity of pMaeWT increased 21-fold after serum stimulation, while the activity of pMaeΔE2F, which contains a mutated E2F element, increased only 5-fold (Fig. 4E). The activities of the dhfr promoters having the B-myb E2F element positioned at nucleotide −375, increased only 4- to 5-fold after serum stimulation (Fig. 4E). These results demonstrate that the B-myb E2F element cannot confer growth regulation from a distance upstream of the core dhfr promoter. Thus, our experiments indicate that the position-dependent S-phase-specific activation of the dhfr promoter by E2F is specified by the core dhfr promoter and not by the E2F element.

The C-terminal transactivation domain of E2F can mediate position-dependent activation of the dhfr promoter. Several properties have been ascribed to the various members of the E2F family of transcription factors. For example, E2F1, E2F2, and E2F3 contain regions near the N terminus that bind to Sp1 and cyclin A (27, 30, 31, 36, 59). E2F1 has been shown to bind DNA (9); the other family members may share this property, since DNA bending is mediated by a region common to all five E2F proteins. Furthermore, all five E2F family members contain activation domains at the carboxyl terminus of the protein (17, 22, 26, 35). To determine which domain of the E2F proteins was responsible for the position-dependent activation of dhfr transcription in S phase, we created a Gal4-responsive dhfr promoter-reporter construct by replacing the E2F element with a Gal4 element (Fig. 5A). This replacement deleted dhfr promoter sequences from positions +20 to +61 and inserted an additional 5 bp between the transcription start site and upstream Sp1 elements. To control for any effects on the position of the start site or transcriptional activity of the promoter, we also created the pseudoWT reporter construct (Fig. 5A), which contains the 5-bp insertion and ends at +20 but still
contains an E2F element. Before analysis of these promoter constructs in cells, the start sites and activity of the p410WT, pseudoWT, and DHFRGal4 constructs were analyzed by in vitro transcription reactions with HeLa cell nuclear extract. As expected due to the loss of dhfr sequences and vector sequences (see Materials and Methods), DHFRGal4 and pseudoWT produced RNA transcripts approximately 61 bp shorter than the RNA transcript produced by p410WT (Fig. 5B). Also, the transcriptional activities of DHFRGal4 and pseudoWT were similar to the activity of p410WT, indicating that the basal levels of transcription for DHFRGal4 and pseudoWT are comparable to that of p410WT.

Our experiments using Gal4-E2F fusion constructs were performed as cotransfection assays using asynchronously growing NIH 3T3 cells. For a control, we examined the ability of all Gal4 fusions to activate pG5TI, a promoter construct containing five Gal4 elements cloned upstream of a TATA box and an initiator element (Fig. 6A). We found that the N terminus of E2F1, when fused to the Gal4 DNA binding domain, could not activate transcription from DHFRGal4 or pG5TI (data not
The E2F transactivation domain contains a special region that allows it to activate the *dhfr* promoter. Our results suggest that growth regulation of the *dhfr* promoter involves position-dependent activation of transcription by the E2F transactivation domain. We next wished to determine if other transactivation domains displayed a similar position dependence with the *dhfr* promoter. We first examined the ability of several Gal4 fusions to activate the pG5TI promoter in asynchronously growing NIH 3T3 cells. Cotransfection of pG5TI or DHFR Gal4 with Gal4-MyoD, Gal4-Ets2, Gal4-YY1 (amino acids 1 to 200 or 1 to 414), Gal4-p53, Gal4-Sp1 (amino acids 83 to 621), Gal4-ATF1, Gal4-ATF2, and Gal4-EBNA1 expression plasmids all resulted in very minimal activation of transcription (data not shown), indicating that these Gal4 fusion proteins are very weak activators of both the pGSTI and *dhfr* promoters. However, we found that the E2F1 C-terminal and VP16 N-terminal transactivation domains are equally potent activators of pG5TI, both resulting in approximately 4,000-fold activation of transcription (Fig. 7A). Therefore, we tested the ability of Gal4-VP16 to activate the *dhfr* promoter from a position at the transcription start site and upstream of the core promoter. Surprisingly, we found that Gal4-VP16 could not activate the *dhfr* promoter from either position (Fig. 7B). Previously, it has been demonstrated that Gal4-VP16 is a much more efficient activator of TATA-containing promoters than TATA-less promoters both in vitro and in vivo (7, 12). The *dhfr* promoter does not contain a consensus TATA element (46); therefore, it is possible that the inability of Gal4-VP16 to activate the *dhfr* promoter is due to the lack of a TATA element. To determine if the addition of a TATA element to the *dhfr* promoter could alter the ability of Gal4-VP16 to activate transcription, we employed a *dhfr* promoter-reporter construct containing a TATA element centered at −30 in the DHFRGal4 promoter. For a control, we also examined the ability of Gal4-E2F1 to activate this promoter. We found that Gal4-E2F1 gave an 85-fold activation of transcription, but Gal4-VP16 gave only a 4-fold activation of transcription (Fig. 7C). These results demonstrate that the inability of the N-terminal transactivation domain of VP16 to activate the *dhfr* promoter is not due to the lack of a TATA element. Taken together, our results suggest that S-phase activation of *dhfr* transcription requires the recruitment of a specific transactivation domain (i.e., that from an E2F family member) to a position proximal to the transcription start site of the *dhfr* promoter.

**DISCUSSION**

Many promoters that contain E2F elements display differential activity in G0 versus S phase. A model for E2F-mediated regulation of transcription has been previously proposed in which G0-phase repression of transcription is conferred by E2F-pocket protein (Rb, p107, or p130) complexes. According to this model, the increase in transcription observed in S phase is due to release of the transcriptional repressor pocket protein from E2F proteins, which occurs upon phosphorylation of the pocket proteins by cyclin-cdk complexes. This model is consistent with mutational analyses of promoters such as B-myb that show constitutively high activity in G0, and S phase upon mutation of the E2F element (33, 34). However, this model does not sufficiently explain the regulation of the *dhfr* promoter. We now propose a model in which growth regulation of *dhfr* transcription involves the S-phase-specific activation of the *dhfr*
promoter by an E2F transactivation domain that must be positioned proximal to the transcription start site. This model is based on the following observations.

(i) Mutation or displacement of the E2F element causes a decrease in S-phase levels of transcription, not an increase in G0-phase levels of transcription. These results are in contrast to studies done on the B-myb promoter which have shown that E2F confers G0-phase repression of transcription (33, 34).

(ii) Movement of the E2F element to an upstream (−375) or downstream (+66) position abolishes growth regulation of the dhfr promoter, indicating that E2F can regulate the dhfr promoter only from a position proximal to the transcription start site. These results are in contrast to other studies showing that an E2F element can confer repression to the SV40 promoter from a distal position (56). We show that two dhfr E2F elements cannot confer growth regulation to the dhfr promoter from a distal upstream position (−375), even though the same two dhfr E2F elements can confer growth regulation when positioned at a distance upstream of the SV40 promoter (52). We also show that the B-myb E2F element, which has been suggested to confer G0-phase repression to the B-myb promoter, is able to confer growth regulation to the dhfr promoter only when located proximal to the transcription start site. Thus, the position dependence of E2F-mediated growth regulation is specified by the core dhfr promoter and not by the characteristics of a particular E2F element.

(iii) The transactivation domains of E2F proteins display position-dependent activity at the dhfr promoter. Other studies have suggested that E2F proteins can be stabilized on promoter DNA by interaction with Sp1 and NFY (27, 36, 55). Although we have changed the distance between the E2F and Sp1 elements in our experiments, we do not believe that DNA binding is specifying position dependence in the dhfr promoter. This is because the DNA binding requirement of E2F for interaction with other proteins can be relieved when binding is mediated via the Gal4 DNA binding domain (55). We note that the Gal4 DNA binding domain has also been shown to bind DNA (45). However, we determined that the Gal4 DNA binding domain alone does not activate the dhfr promoter (data not shown). Thus, the position-dependent activation is mediated by a property inherent to the Gal4-E2F fusion proteins that is not present in the Gal4 DNA binding domain. We do not rule out the involvement of Sp1 or DNA bending in the regulation of the endogenous dhfr promoter. However, the use of a heterologous DNA binding domain has allowed us to

FIG. 6. The transactivation domains of E2F1, E2F4, and E2F5 show position-dependent activation of the dhfr promoter. The ability of the Gal4-E2F fusion proteins to activate the p63T1 (A), DHFRGal4 (B), and DHFR-Gal4@−375 (C) reporter constructs was examined in transiently transfected asynchronously growing NIH 3T3 cells. Each reporter plasmid and Gal4 fusion expression plasmid (5 μg of each) were transfected into 10⁵ cells, which were then incubated in growth medium for 48 h. Schematics of the p63T1, DHFR-Gal4, and DHFRGal4@−375 reporter constructs are shown above each graph. Luciferase values are reported as a ratio of the activity of each reporter construct in the presence of the specified Gal4 fusion expression plasmid relative to the activity of the same reporter construct in the presence of the Gal4(1-147) expression plasmid. Bars represent standard errors of the means.
examine the post-DNA-binding requirements for E2F-mediated activation of \textit{dhfr} transcription, and our results suggest that the transactivation domain alone conveys the dependence on position.

Our model requires that an E2F transactivation domain be brought near the \textit{dhfr} transcription start site for high-level activity from the \textit{dhfr} promoter (Fig. 8). Since E2F proteins are bound to at least one strand of the \textit{dhfr} promoter throughout the cell cycle, but \textit{dhfr} transcription levels are high only in S phase, interactions between the core \textit{dhfr} promoter and E2F must be productive only in S phase. We suggest that the \textit{dhfr} promoter has the potential to be responsive to the E2F transactivation domain throughout the cell cycle but that low promoter activity in G\textsubscript{0} phase is due to the masking of the critical E2F activation domain by the pocket proteins. This masking is distinguished from the repression of a strong promoter (such as B-myb) because the \textit{dhfr} promoter is not active in the absence of an E2F protein. Thus, unlike other promoters, the increase in activity of the \textit{dhfr} promoter in S phase requires the presence of an active E2F complex; it is not sufficient to simply release a transcriptional repressor pocket protein.

We have shown that the \textit{dhfr} promoter has a special requirement for the transactivation domain of E2F proteins that is not observed in most E2F-regulated promoters. This critical requirement of the \textit{dhfr} promoter for transactivation by E2F proteins is emphasized by the fact that the potent N-terminal transactivation domain of VP16 cannot substitute for the E2F activation domain. Several properties have been ascribed to the E2F transactivation domain, including binding to the TATA-binding protein, TFIIH, the CREB-binding protein, and the Rb family of pocket proteins (3, 13, 26, 54). However, the N-terminal transactivation domain of VP16 has been shown to bind TFIIH and TFIIIB (24, 37), suggesting that the binding of TFIIH and/or TFIIIB is not sufficient for the activation of the \textit{dhfr} promoter. We have determined that an E2F1 transactivation domain shown to be deficient in Rb binding (17) can still efficiently activate the \textit{dhfr} promoter (data not shown), suggesting that the binding of Rb is not critical for
activation of dhfr transcription (although it may play a role in masking the E2F transactivation domain in G0 phase). It remains possible that the binding of TFIIH and/or CREB-binding protein by E2F is critical for the activation of dhfr transcription. For example, it has previously been shown that transcription from the dhfr promoter in vitro requires the C-terminal domain (CTD) of the largest subunit of RNA polymerase II and the CTD-kinase activity of TFIIH, suggesting that dhfr transcription may be regulated at the level of promoter clearance (1, 6). Thus, it is possible that E2F activates the dhfr promoter by recruiting or stimulating the TFIIH CTD-kinase activity and increasing levels of promoter clearance.

In summary, we have provided evidence that the dhfr promoter is highly active only when an E2F transactivation domain is positioned at the transcription start site and unmasked from the pocket proteins. We suggest that the E2F transactivation domain binds a specific factor that is critical for the activation of the dhfr promoter. Further experiments will allow us to identify the factor required for dhfr activation and characterize the mechanism by which E2F activates transcription.

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REFERENCES


32. van Ginkel, P. R., K.-M. Hsiao, H. Schjerven, and P. J. Farnham. Submitted for publication.