

## The Use of Transient Chromatin Immunoprecipitation Assays to Test Models for E2F1-specific Transcriptional Activation\*

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**The E2F family of transcription factors regulates the expression of genes involved in cell cycle progression, DNA synthesis, repair, and recombination, and a variety of other cellular processes. Although E2F proteins are often redundant in function, specificity of binding and activity can occur. For example, E2F1, but not other E2F family members, was shown previously to bind the murine carboxylesterase promoter in chromatin immunoprecipitation studies (Wells, J., Graveel, C. R., Bartley, S. M., Madore, S. J., and Farnham, P. J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 3890–3895). This promoter region lacks a consensus E2F binding site, suggesting that E2F1 may be recruited to the DNA in a unique fashion. To further investigate this E2F1-specific binding, we have employed a “transient chromatin immunoprecipitation” approach. Using various deletions and mutations of the promoter region, we localized the E2F1-specific binding site and demonstrated that it was required for E2F1-mediated transcription of the carboxylesterase promoter. The identified site was similar to the 8-bp consensus E2F site but differed from the consensus at a crucial position. To address whether E2F1 directly bound to this non-consensus site, we demonstrated that the DNA binding domain of E2F1 is necessary for E2F1-mediated activation of the carboxylesterase promoter. Interestingly, a “UP” mutation of the site, making it more similar to the consensus element, did not improve the ability of E2F1 to bind the promoter. Rather, E2F1 could no longer bind to the carboxylesterase promoter that contained the consensus E2F site. We propose a model in which E2F1-specific regulation of the carboxylesterase promoter requires both E2F1/DNA interactions and protein-protein interaction between E2F1 and a factor that binds adjacent to the non-consensus site.**

The E2F transcription factors regulate the expression of genes involved in cell cycle progression, DNA synthesis and repair, and various other functions (1). The E2F family consists of nine members; E2F1 through E2F6 form heterodimers with DP1 or DP2 to bind DNA, whereas E2F7 can bind DNA via a DP-independent mechanism (2–4). The E2F-DP heterodimers bind DNA in a sequence-specific fashion, resulting in transcriptional activation or, when complexed with a pocket protein such as pRB, transcriptional repression (2). Although the E2F proteins are quite similar to each other, it has been demonstrated

that these proteins do have distinct roles in the cell. For example, E2F3 is necessary for the G<sub>1</sub> to S phase transition, whereas E2F1 is involved in the apoptotic response and may also play a role in sensing DNA damage (5–9). Additionally, it has been demonstrated that the loss of a single E2F family member results in distinct effects in mouse models. For instance, loss of E2F1 leads to increased susceptibility to tumor formation in various mouse tissues and decreased T cell apoptosis, whereas E2F5 null mice die of hydrocephaly soon after birth (10–12).

The mechanism by which E2F proteins carry out their individual functions is not yet understood. Although one possibility is that different E2Fs might bind to different promoters, chromatin immunoprecipitation (ChIP)<sup>1</sup> assays have shown that most E2F target genes are bound by multiple E2Fs (6, 13, 14). However, several recent studies using ChIP assays have led to the discovery of a few promoter regions that are bound and regulated by a single E2F family member. For example, to identify genes regulated specifically by E2F1, oligonucleotide microarrays were used to compare mRNA expression levels in the livers of wild type and E2F1 nullizygous mice. Very few genes changed their expression upon the loss of E2F1. However, a few mRNAs had reduced expression in the mutant mice, implicating E2F1 in their activation. Accordingly, ChIP assays showed that E2F1 was bound to these promoter regions. As would be predicted by the sensitivity of these genes to the loss of a single E2F, the promoters were bound exclusively by E2F1 and not by other E2F family members in mouse liver, kidney, spleen, and testes tissues (15).

We have now undertaken a detailed analysis of one of the E2F1-specific promoters, carboxylesterase. The carboxylesterase enzyme, a member of the multigene family EC 3.1.1.1 of serine hydrolases, is expressed predominately in the liver and is involved in detoxification of various drugs and carcinogens, as well as cellular compounds important for metabolism (16). We show here that an E2F site similar to but divergent from the consensus site is responsible for E2F1-specific binding and transcriptional activation of the carboxylesterase promoter.

### EXPERIMENTAL PROCEDURES

**Cell Culture**—293 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). NIH3T3 cells were grown in Dulbecco's modified Eagle's medium with 10% bovine calf serum (Invitrogen) and 1% penicillin-streptomycin.

**Plasmid Constructs**—All carboxylesterase promoter deletions were cloned into the luciferase reporter vector pAAlucA. Carboxylesterase promoter constructs were created via PCR using NIH3T3 DNA as a template and primer +25Carb (containing a HindIII site) with various upstream primers (each containing a SpeI site). Primers used are listed in Tables I and II and were synthesized at the University of Wisconsin Biotechnology Center. The PCR products were cleaned using the Qiaquick PCR purification kit (Qiagen, Valencia, CA), cut with SpeI and

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<sup>1</sup> The abbreviation used is: ChIP, chromatin immunoprecipitation.

TABLE I  
Primers for cloning

Orientation	Name	Sequence (5' to 3')
Forward	Wild type	GGA CAT ACT AGT CTA GTT CAC TCT CTG CTG TG
	-650Carb	GGA CAT ACT AGT GCT CTA TGC GTT TCA TCA GA
	-590Carb	GGA CAT ACT AGT CTG CTC CAA AAG CAG TGA GAT
	-530Carb	GGA CAT ACT AGT CAG GCA AGG ATA GTT GGC AT
	-380Carb	GGA CAT ACT AGT GAG TAA ACT CGT AGT GGG TG
	-570Carb	GGA CAT ACT AGT CAT TAA CAG AGA AGG TGT GC
	AKOCarb	GGA CAT ACT AGT ACA TCC CAG ATC TAA AAG CAG TGA G
	BKOCarbA	GAA GGT GTG CAA GAT CTC TTC AGG CCA
	AUPCarb	GGA CAT ACT AGT TTA AAC ATC CCT GCG CCA AAA GCA GTG
	+25Carb	AGC GAA GCT TAA GAG AAG CCC AGA CCA GAA C
Reverse	BKOCarbB	TGG CCT GAA GAG ATC TTG CAC ACC TTC

TABLE II  
Primers for ChIP analysis

Use	Orientation	Sequence
Plasmid ChIP	Forward	TGC TCT TAA TTC TCC AGC TGG
	Reverse	CTC TTC ATA GCC TTA TGC AGT
Endogenous <i>dhfr</i>	Forward	TTC TGC TGT AAC GAG CGG GCT CGG A
	Reverse	CTA CAA GTT AGA GAA ACA GCG TTA CTC GAA
3'-End endogenous <i>dhfr</i>	Forward	CTG ATG TCC AGG AGG AGA AAG G
	Reverse	AGC CCG ACA ATG TCA AGG ACT G

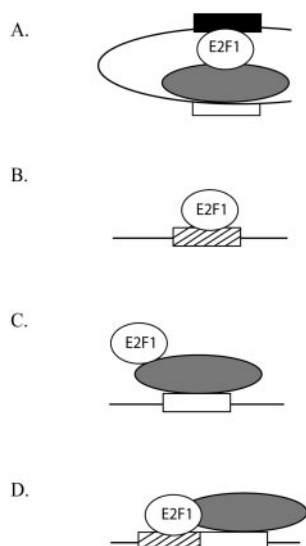
HindIII (New England Biolabs, Inc., Beverly, MA), and cloned into the same sites in the pAAlucA vector. Successful cloning of the various inserts was confirmed by sequencing at the University of Wisconsin Biotechnology Center. To create the AKO- and AUP-CARB constructs, mutagenic primers were used (see Table I). To create the BKO-CARB construct, a two-step overlap PCR technique was used. Briefly, one PCR reaction was performed using primers BKOCarbA and +25Carb, and a separate reaction was performed using the primers -650Carb and BKOCarbB. PCR products were cleaned and combined to use as template for a final PCR reaction with the flanking primers -650Carb and +25Carb. This product was cleaned, cut with restriction enzymes, and cloned as described above. Other plasmids used in this study, including pcDNA3, pCMV E2F1 wild type, and pCMV E2F1 E138 have been described previously (17).

**Chromatin Immunoprecipitation Assays with Transiently Transfected Cells**—293 cells were grown overnight in 100-mm dishes to ~60–70% confluency; cells were then transfected with 1  $\mu$ g of the promoter construct using a FuGENE 6 transfection reagent (Roche Applied Sciences). Plates were returned to the incubator for 40–48 h. At this time, cells were cross-linked with formaldehyde, harvested, and chromatin immunoprecipitations were performed. For these studies, only  $5 \times 10^6$  cells were used per immunoprecipitation reaction because the plasmid is present at a high copy number. The remainder of the procedure followed standard protocols for ChIP analysis, as has been published previously (18–20) and described on the University of California at Davis Genome Center web site ([genomecenter.ucdavis.edu/farnham/](http://genomecenter.ucdavis.edu/farnham/)). The resulting DNA was analyzed by PCR reactions with a forward primer to a downstream portion of the carboxylesterase promoter that is common to all constructs and a reverse primer to the pAAlucA plasmid backbone (denoted plasmid ChIP forward and reverse primers are shown in Table II). Additionally, each ChIP sample was also subjected to PCR with primers to the *dhfr* promoter (a positive control) and the 3'-end of the *dhfr* gene (a negative control). Antibodies used in the ChIP procedure include (from Santa Cruz Biotechnology, Inc. Santa Cruz, CA) E2F1 (catalog number sc-193X), E2F2 (catalog number sc-633X), E2F3 (catalog number sc-878X), E2F4 (catalog number sc-866X), E2F5 (catalog number sc-999X), and E2F6 (catalog number sc-8366) as well as rabbit anti-mouse IgG.

**Luciferase Assays**—Approximately  $2 \times 10^5$  NIH3T3 cells were grown overnight in 60-mm dishes and transfected the next day using calcium phosphate with 2  $\mu$ g of reporter constructs (-590CARB or -530CARB), 2  $\mu$ g of expression vector (pCMV E2F1 wild type, pCMV E2F1 E138, or pcDNA3), and 11  $\mu$ g of sonicated salmon sperm. Six hours after transfection, the media were changed to starvation media (Dulbecco's modified Eagle's media supplemented with 0.5% bovine calf serum and 1% penicillin-streptomycin) and incubated for 45–60 h. Luciferase assays were then performed as described previously (21). Results presented are the average of quadruplicate experiments with the S.D. values shown as error bars (Fig. 4).

## RESULTS

In our previous studies, we demonstrated that the loss of E2F1 results in a reduction in the levels of mouse carboxylesterase mRNA and that the carboxylesterase promoter is bound by E2F1, but not by other E2F family members, in mouse liver, kidney, and testes tissue (15). The requirement for E2F1 for robust expression of carboxylesterase mRNA and the extreme selectivity of the binding of E2F1 to the carboxylesterase promoter suggest a crucial role for E2F1 in regulation of the carboxylesterase gene. However, an examination of the carboxylesterase promoter region revealed no consensus binding sites for E2F proteins. It is important to note that our previous ChIP studies were performed using wild type mice that expressed all of the E2F family members at physiologically normal levels; thus, the selectivity of binding was not due to artificial overexpression of E2F1. Rather, our results suggested one of four possible models of E2F1-mediated regulation of the carboxylesterase gene (Fig. 1). First, it is possible that E2F1 binds to a consensus site located at a distance from the start site of transcription and cross-links to the proximal promoter region because of a looping mechanism. Second, E2F1 may bind, via its DNA binding domain, to a non-consensus E2F site in the proximal promoter region. Third, E2F1 may be indirectly associated with the DNA via interaction with another DNA-binding protein. Fourth, recruitment of E2F1 to the carboxylesterase promoter may involve both E2F1 binding to the DNA and protein-protein interactions. To distinguish these models, it was necessary to localize the cis element that mediates E2F1-specific activation of the carboxylesterase promoter. Clearly, if multiple proteins and/or chromatin structure is involved in E2F1-specific activation, methods such as gel shifts, which rely on small oligonucleotides and artificial buffer conditions, may not be appropriate. Because an *in vivo* assay was required, we used a "transient ChIP" assay. This method involves cloning the promoter of interest (and mutant derivatives) into a plasmid, transiently transfecting the plasmid into a cell line, and then assaying the ability of various proteins to bind this sequence *in vivo* using chromatin immunoprecipitation assays (Fig. 2A). For the transient ChIP experiments, a cell line must be used that is efficiently transfected, easily amenable to ChIP assays, and contains the transcription factor of interest, because overexpression of the transcription factor



**FIG. 1. Models of E2F1-specific gene regulation at the murine carboxylesterase promoter.** Models for E2F1-specific binding and subsequent transcriptional activation of the murine carboxylesterase promoter are outlined. *A*, E2F1 binds at a distance and interacts with the basal promoter via a looping mechanism. E2F1 is shown as an *open circle*, and the E2F binding site is shown as a *filled rectangle*. An unknown transcription factor is shown as a *shaded oval*, with its binding site shown as an *open rectangle*. *B*, E2F1 binds a non-consensus site. E2F1 is shown as an *open circle*, and the non-consensus site is shown as a *hatched rectangle*. *C*, E2F1 indirectly binds DNA via association with another DNA-binding protein. E2F1 is shown as an *open circle*, the second factor is shown as a *shaded oval*, and the binding site for the second factor is shown as an *open rectangle*. *D*, E2F1 is recruited to the DNA via protein-protein interactions, and the interaction of the complex with the promoter region is stabilized by E2F1 binding to a non-consensus site. E2F1 is shown as an *open circle*, the non-consensus E2F site is shown as a *hatched rectangle*, the second factor is shown as a *shaded oval*, and the binding site for the second factor is shown as an *open rectangle*.

may yield false positive results. Because we are studying the mouse carboxylesterase promoter, we first attempted to use the well characterized NIH3T3 cells for the transient ChIP assays. However, we have shown previously that E2F1 is difficult to detect on endogenous E2F target genes in asynchronously growing NIH3T3 cells (13). This difficulty, combined with the inefficiency of transfection of these cells, resulted in our inability to develop a transient ChIP assay for NIH3T3 cells. Therefore, we have used human 293 cells to study the carboxylesterase promoter because they transfect well, work well in ChIP assays, and easily demonstrate the binding of E2F1 to endogenous promoters.

**E2F1-specific Binding Is Maintained on the Carboxylesterase Promoter in Transient ChIP Assays**—Our first set of experiments was designed to determine whether the E2F1-specific binding site could be localized to the proximal promoter region. If so, then E2F1 recruitment to a distant consensus site could be eliminated as a possible mode of regulation of the carboxylesterase promoter. To determine whether E2F1 specificity is mediated by the proximal promoter, an 800-bp carboxylesterase promoter region was cloned into the reporter vector pAALucA. After transfection into 293 cells, the binding of endogenous E2F proteins was tested (Fig. 2*B*). A strong PCR signal was seen when an anti-E2F1 antibody was used. Little to no signal was seen with other antibodies, including IgG, which was used as a control for nonspecific precipitation of protein-DNA complexes. The samples were also subjected to PCR reactions using primers to the endogenous *dhfr* promoter to ensure that the immunoprecipitations were indeed successful, as multiple E2F proteins are known to associate with this

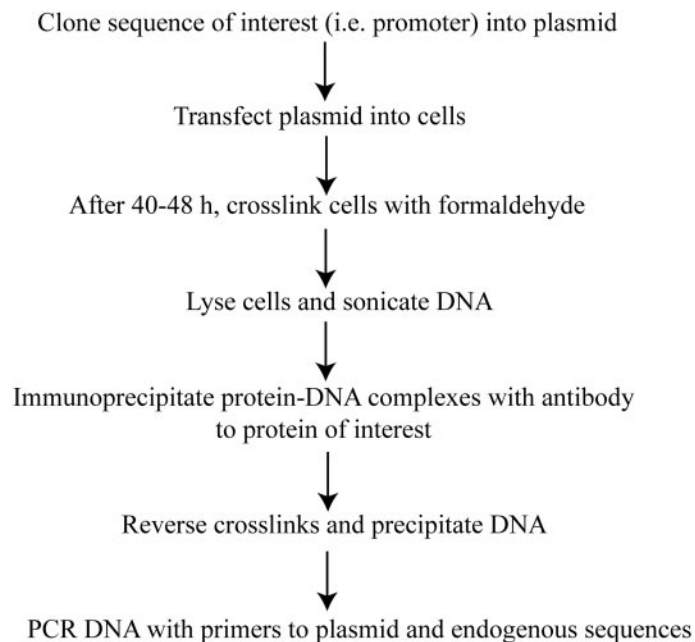
region (13). As predicted, E2F1–4, but not E2F5 or E2F6, bound to the *dhfr* promoter. As a negative control, PCR reactions were performed with primers to the 3'-end of the *dhfr* gene, and, as expected, no signal was seen for the immunoprecipitated samples. These results demonstrate that E2F1 is directly recruited to the proximal carboxylesterase promoter region, eliminating Model A (binding of E2F1 at a distance in combination with looping). Importantly, these results also demonstrate that, in the presence of physiologically normal levels of E2F1, the binding specificity that we originally observed by monitoring the endogenous carboxylesterase promoter is maintained on the transiently transfected plasmid.

**E2F1-specific Activation Requires the DNA Binding Domain of E2F1**—Our previous work had shown that elimination of E2F1 from the cell caused a reduction in endogenous carboxylesterase mRNA, indicating that E2F1 activates the carboxylesterase promoter (15). To distinguish whether direct or indirect recruitment of E2F1 is responsible for the increase of carboxylesterase mRNA levels, we wanted to compare the ability of wild type E2F1 *versus* E2F1 having a non-functional DNA binding domain to activate the carboxylesterase promoter. However, promoter-reporter assays are best interpreted when constructs containing and lacking a specific cis element are compared. Because we did not know which region of the carboxylesterase promoter was critical for E2F1-mediated regulation, we first localized the E2F1 binding site using the transient ChIP assay. To map the binding site for E2F1, deletions were made throughout the upstream carboxylesterase promoter sequence. Four deletions of the upstream sequence were created, and each construct was tested for *in vivo* binding of endogenous E2F1 using the transient ChIP assay (Fig. 3). For the constructs –650CARB and –590CARB, which remove 150 and 210 bp of the upstream sequence, respectively, E2F1 binding was retained. However, the deletion of just 60 bp more resulted in a loss of binding (see the –530CARB row in Fig. 3), narrowing down the E2F1 binding site to a 60-bp region. As expected, a more drastic deletion (–380CARB) was also no longer bound by E2F1. For each transient ChIP experiment, binding of E2F1 to the *dhfr* promoter, but not to the 3'-end of the *dhfr* gene, was confirmed.

Having localized the E2F1 binding site, we next confirmed that the identified region is responsible for the E2F1-mediated regulation of the carboxylesterase promoter. The role of this E2F1 binding region in transcriptional activation was tested in luciferase assays using the constructs –590CARB (which contains the E2F1 binding site) and –530CARB (which lacks the binding site). As seen in Fig. 4, the activity of the –590CARB construct was indeed higher when a plasmid expressing E2F1 was transfected into the cells, as compared with vector alone. Importantly, this activation did not occur when the –530CARB construct was used. Finally, we tested whether the ability of E2F1 to bind to DNA was required for the activation. An E2F1 construct that has been shown previously to lack DNA binding activity because of a two-amino acid change in the DNA binding domain was unable to activate the –590CARB construct. Taken together, the ChIP studies and the promoter-reporter studies indicate that our transient transfection system accurately mimics both E2F1-specific binding and E2F1-mediated regulation of the endogenous carboxylesterase promoter.

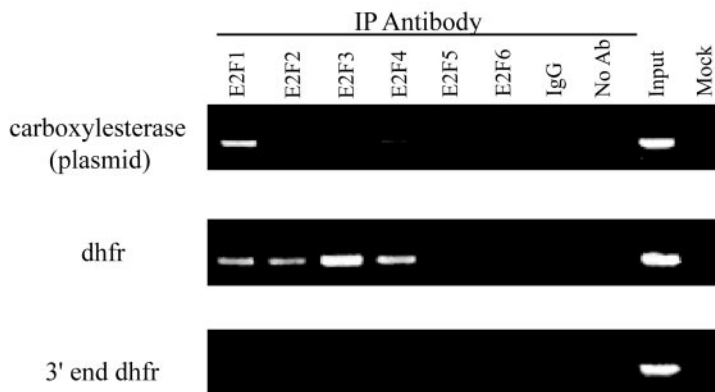
**E2F1 Is Recruited to the Carboxylesterase Promoter via an E2F-like Site**—The requirement for the E2F1 DNA binding domain for E2F1-mediated activation of the carboxylesterase promoter suggests that E2F1 may be recruited to the DNA via an element that resembles an E2F consensus site. Examination of the 60-bp region mapped above revealed two putative E2F binding sites that differ from the consensus by only one or two

A.

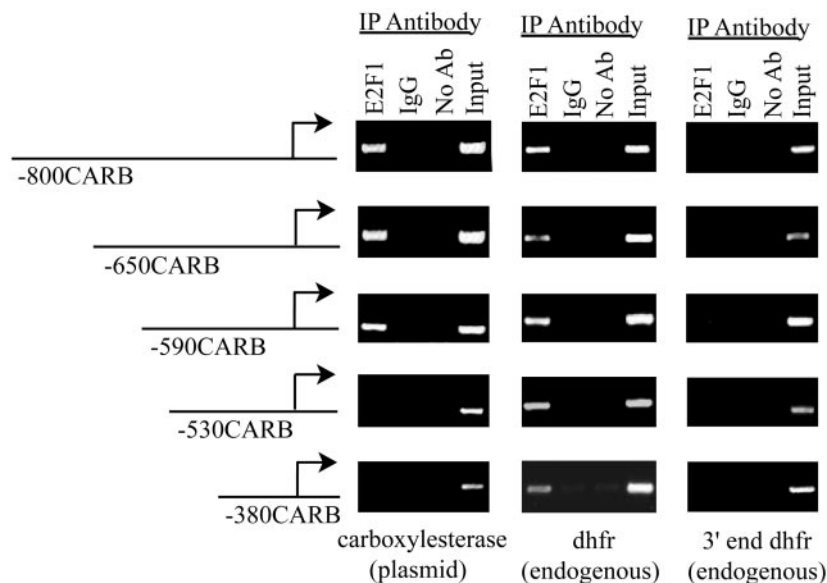


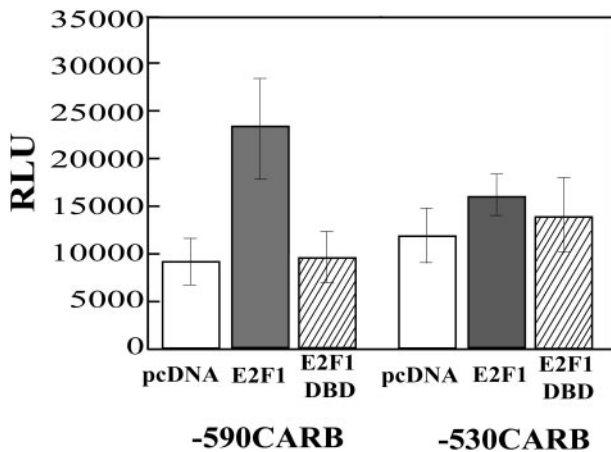
**FIG. 2. Development of a transient ChIP assay that allows E2F1-specific binding to the murine carboxylesterase promoter.** A, the protocol for the transient ChIP assay used throughout this paper is outlined. B, transient ChIP assays were performed with 293 cells transfected with a plasmid containing the murine carboxylesterase promoter. Chromatin was isolated from cells containing the construct  $-800\text{CARB}$  (consisting of an 800-bp fragment of the carboxylesterase promoter cloned into plasmid pAAlucA), and immunoprecipitations were performed with antibodies to E2F1 through E2F6 and a control IgG. PCR reactions were carried out with the various ChIP samples along with a “no antibody” control (*No Ab*) that contains chromatin but no antibody, an “input” sample that represents 0.2% of the total input chromatin, and a “mock” sample that does not contain chromatin. Primers in the plasmid were used in the *top panel*. In the *middle panel* primers to the endogenous *dhfr* promoter were used as a positive control for the immunoprecipitations, and in the *bottom panel* primers to the 3'-end of the endogenous *dhfr* gene were used as a negative control.

B.



**FIG. 3. Transient ChIP assays of carboxylesterase promoter deletions.** Deletions of the carboxylesterase promoter region were created and cloned into pAAlucA as described under “Experimental Procedures.” Immunoprecipitations (*IP*) and PCR reactions were performed as described in Fig. 2 using an antibody to E2F1 or a negative control antibody (IgG). As another negative control, a sample with no added antibody was also tested (*No Ab*), and an input sample is also included. At the *far left*, a schematic of the constructs created is shown. To the *right*, the results of PCR reactions using the various immunoprecipitated samples are shown. The *left column* represents PCR reactions with primers to the carboxylesterase promoter plasmid construct, the *middle column* represents PCR reactions with primers to the endogenous *dhfr* promoter (positive control), and the *right column* represents PCR reactions with primers to the 3'-end of the endogenous *dhfr* gene (negative control).





**FIG. 4. E2F1 requires DNA binding activity to activate the carboxylesterase promoter.** Asynchronously growing NIH3T3 cells were cotransfected with 2  $\mu$ g of either -590CARB or -530CARB promoter constructs and 2  $\mu$ g of pcDNA3 (*pcDNA*), pCMV E2F1 wild type (*E2F1*), or pCMV E138, a DNA binding mutant (*E2F1 DBD*). Luciferase assays were performed in quadruplicate, and the results are shown in graphical presentation as an average of these experiments with the S.D. shown as *error bars* and numerical values at the *left* given as relative light units (*RLU*). The transfection and luciferase protocols are described under "Experimental Procedures."

base mismatches (Fig. 5, shown as *Site A* and *Site B*). However, it is important to note that the differences are in nucleotides critical for high affinity E2F binding (22, 23). Mutations of these E2F-like sites were created to determine whether one or both are responsible for E2F1-specific binding. A deletion removing the upstream binding site, Site A, was created (-570CARB); E2F1 binding was lost in transient ChIP assays (Fig. 5A). Additionally, point mutation of Site A also resulted in a loss of E2F1 binding (AKO-CARB). It was possible that Site B was also required for binding, in addition to Site A. To determine whether the second putative E2F binding site was necessary but not sufficient for E2F1 recruitment, BKO-CARB (which alters Site B without changing Site A) was created. When this construct was tested in transient ChIP assays, E2F1 binding was maintained, demonstrating that the downstream site is not necessary for E2F1 binding. Thus, the upstream Site A mediates E2F1-specific binding.

As noted above, the site required for E2F1-specific binding resembles the E2F consensus TTT(C/G)(C/G)CGC. However, Site A lacks the critical G or C at position 6. To further explore the sequence requirements for the binding of E2F1 to the non-consensus site in the carboxylesterase promoter, a mutation was created that changed the T at position 6 to a G, thus creating a consensus E2F site (AUP-CARB; Fig. 5B). Surprisingly, we found that the mutation toward consensus resulted in the loss of binding by E2F1, demonstrating that E2F1 requires the non-consensus site for binding at the carboxylesterase promoter.

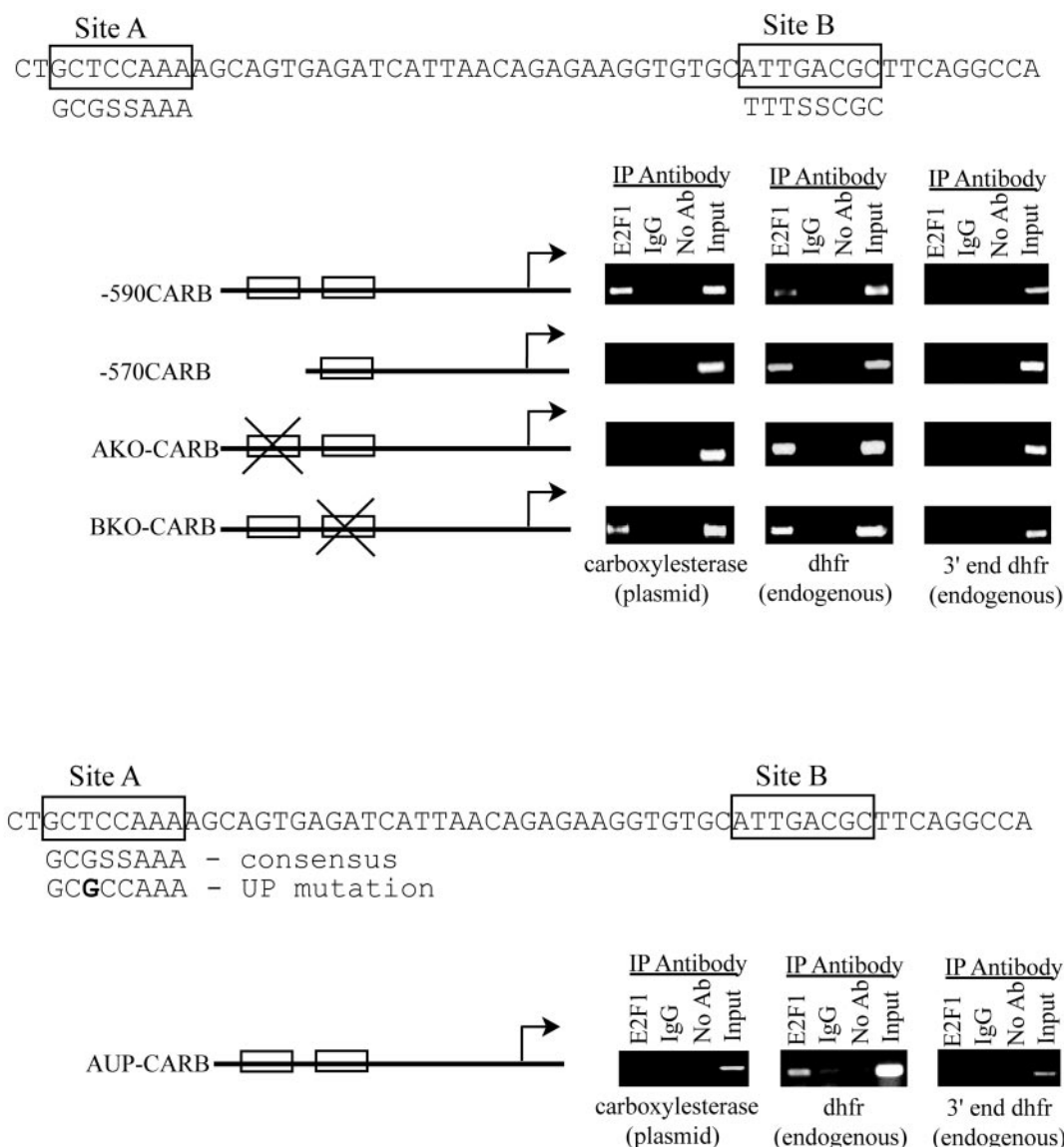
**Other E2Fs Cannot Bind to Mutated Carboxylesterase Promoters**—The above results show that E2F1 is recruited via an E2F-like site to the carboxylesterase promoter. However, it is curious that other E2Fs cannot bind to either of the E2F-like sites. It was possible that the upstream site was responsible for the specificity of E2F1 binding to the promoter and that deletion of that site would allow other E2Fs to bind to the downstream site, or *vice versa*. To test that possibility, transient ChIP assays were performed using antibodies to E2F1, E2F2, and E2F4 (Fig. 6). For the deletion construct -590CARB, the shortest deletion that still contains Site A, only E2F1 bound the promoter. Specificity of E2F1 binding was also seen with the mutation of Site B; *i.e.* Site A binds E2F1, but not other

E2Fs, in the presence or absence of Site B. Also, mutation of Site A did not allow binding of E2F1 or other E2Fs to Site B. Therefore, Site A is required for E2F1-specific binding, and Site B is not capable of binding any of the E2Fs in the presence or absence of E2F1 bound to Site A. Additionally, the AUP-CARB mutation was tested for binding by other E2F family members, with the expectation that they may be able to bind the newly created consensus site. However, E2F2 and E2F4 also did not bind to the newly created consensus sequence. These results clearly demonstrate that *in vivo* binding of E2F family members cannot be predicted by simply examining a promoter sequence.

## DISCUSSION

Although closely related in structure and activity, E2F family members have distinct roles in the cell. This is especially true for E2F1, the main E2F protein implicated in regulating genes for apoptosis and the DNA damage response (6–9). The search for E2F1-specific target genes has been difficult because of the fact that many studies have shown that most E2F target genes are bound and regulated by multiple E2F family members (6, 13, 14). However, by combining the use of E2F1 nullizygous mice, oligonucleotide-based expression arrays, and chromatin immunoprecipitation, we have found that E2F1, but not other E2Fs, can specifically bind to and regulate certain cellular promoters. For example, we had shown previously that E2F1 could bind to the murine carboxylesterase promoter in various mouse tissues (15). However, unlike most E2F target genes, other E2Fs were not detected at the carboxylesterase promoter using ChIP assays. Also, unlike most E2F target promoters, this promoter is not a CpG island. Finally, we could not identify a consensus E2F site within the proximal promoter region. Therefore, our goal in this current study was to determine the molecular mechanisms through which the E2F1-specific binding and regulation of the carboxylesterase promoter is achieved.

We began by testing four models that could account for the recruitment of E2F1 to a promoter lacking a consensus binding site. These are as follows: (a) binding of E2F1 at a distance in combination with looping (Model A); (b) recruitment of E2F1 via the E2F DNA binding domain to a site that does not match the consensus sequence (Model B); (c) recruitment of E2F1 solely via interaction with another DNA-binding protein (Model C); and (d) recruitment of E2F1 via protein-protein interactions with stabilization of the complex on the DNA via E2F1/DNA interactions (Model D). Using a transient ChIP assay, we could demonstrate that E2F1 binds to the proximal region of the carboxylesterase promoter, clearly ruling out the possibility that the promoter is regulated by E2F1 binding at a great distance from the start site at a consensus E2F element. We also demonstrated that the DNA binding domain of E2F1 is necessary for E2F1-mediated activation of the carboxylesterase promoter and that E2F1 binds directly to a sequence that is similar to (but differing in critical positions from) a consensus E2F binding site. These results, which indicated that E2F1-mediated regulation of the carboxylesterase promoter requires direct binding to a non-consensus E2F site located ~580 bp upstream of the transcription start site, supported either Model B or Model D. However, altering the non-consensus site to a consensus site eliminated recruitment of E2F1, suggesting that a simple E2F/DNA interaction (Model B) was not the mechanism by which E2F1 regulated the carboxylesterase promoter. Because E2F1 could not interact with a consensus sequence at this promoter, we believe that other proteins and/or sequences are involved in the recruitment of E2F1 to the carboxylesterase promoter. Therefore, our results suggest that



**FIG. 5. Fine mapping of the E2F1 binding site at the carboxylesterase promoter.** *A*, shown is the 60-bp E2F1-binding sequence mapped in the deletion studies shown in Fig. 3. Two putative E2F binding sites are boxed; the upstream site is denoted *Site A* and the downstream site is denoted *Site B*. Below the two putative sites, the E2F consensus sequence is shown with *S* representing a G or a C. The results of transient ChIP assays with the various mutant constructs are also shown. To the left, a schematic of the constructs created are shown; boxes represent the putative E2F1 binding sites, and an X through the site represents mutation of the site. To the right, PCR reactions of the immunoprecipitations (IP) are presented. PCR reactions with primers to the promoter constructs are presented on the left, positive control experiments with primers to the endogenous *dhfr* promoter are presented in the middle, and negative controls with primers to the 3'-end of the endogenous *dhfr* gene are presented on the right. *B*, the 60-bp E2F1 binding region is shown. Site A is boxed; below it the E2F consensus and the sequence of the "UP" mutation is shown, with the bases changed shown in boldface. Below, transient ChIP assays were performed with the UP mutation. A schematic of this construct is shown on the left, and the results of the PCR reactions to the various immunoprecipitation samples are shown on the right as described for part A.

Model D most accurately reflects regulation of the carboxylesterase promoter.

The localization of the cis element required for E2F1-specific binding to the carboxylesterase promoter to a non-consensus site confirms the need to study DNA/protein interactions in living cells. Although programs have been developed to identify E2F sites in sets of mammalian promoters (23), there are serious concerns that arise when one relies solely upon a bioinformatics approach to identify binding sites. First, we have shown previously that consensus E2F sites are not always occupied *in vivo*. For example, a consensus binding site for E2F, which is located in exon 2 of the *myc* gene, is not occupied *in vivo* (19). Also, as shown in this current study, the introduction of a promoter that has a consensus E2F site into the cell does not always result in recruitment of E2Fs to that site. It is

possible that replacement of the non-consensus E2F site in the carboxylesterase promoter with a consensus site altered the interaction of a second factor with the promoter, occluding the E2F site and preventing binding of any E2F. Unfortunately, there are no consensus sites for known DNA-binding factors near to the non-consensus E2F site that would likely interact with E2F1. Therefore, we cannot perform ChIP assays with antibodies to putative interacting partners to test this possibility. However, our studies clearly indicate that a bioinformatics approach can lead to the false identification of a promoter as an E2F target. Conversely, many true target promoters may be overlooked if only consensus sites are considered. In computer-assisted comparisons of known E2F binding sites, substitutions of an A or T at position 6 do not occur (23). Moreover, sites identified in cyclic amplification and selection of targets

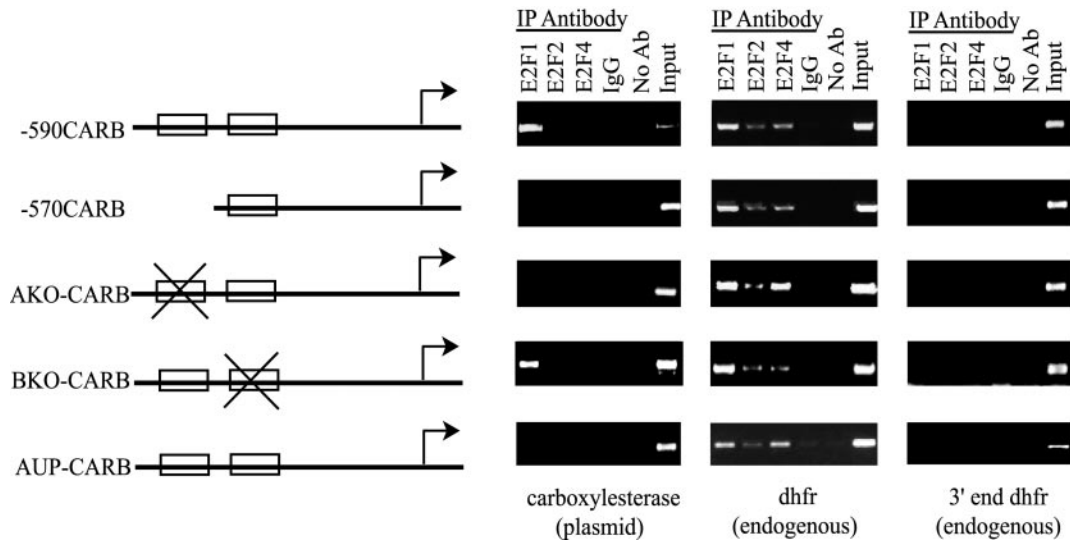


FIG. 6. **Other E2Fs cannot bind to the carboxylesterase promoter in the presence or absence of E2F1 bound to Site A.** Transient ChIP assays were performed with the indicated binding site mutants. To the left the various mutant constructs are shown. To the right the results of PCR reactions using the various immunoprecipitation samples are shown. Immunoprecipitations (IP) were performed with antibodies to E2F1, E2F2, E2F4, and IgG (a negative control). As another negative control, PCR was performed using a no antibody sample (No Ab). A sample containing 0.2% total input chromatin was also included. PCR reactions with primers to the constructs are presented on the left, positive control experiments with primers to the endogenous *dhfr* promoter are presented in the middle, and negative controls with primers to the 3'-end of the endogenous *dhfr* gene are presented on the right.

(CASTing) studies using E2F1 did not contain a mismatch at the position 6, indicating that E2F1 does not bind this type of element with high affinity *in vitro* (22). Thus, as shown here and in other recent studies (24, 25), target genes that contain sites that differ in critical positions from the consensus will be overlooked by identification schemes that rely solely upon the presence of a high affinity consensus site in a promoter region. Finally, we feel that *in vitro* experimental methods are not appropriate for the study of non-consensus binding sites. For example, gel shift assays using small oligonucleotide probes are not optimal for studying the association of protein complexes with DNA if secondary factors are required. For example, the interaction between YY1 and E2F2 is believed to allow stable recruitment of E2F2 to the *cdc6* promoter (26) whereas the interaction of E2F3 and TFE3 is critical in determining E2F3-specific target genes (27, 28). Such interactions would be difficult to study using *in vitro* gel shift assays. Clearly, experimental methods that can allow the delineation of binding site *in vivo* are required. As demonstrated here, we believe that the use of the transient ChIP assay is a rapid, *in vivo* method for localizing transcription factor binding sites on target promoters. The use of such an assay has provided insight into the mechanism by which E2F1 regulates the carboxylesterase promoter and has allowed a deeper understanding of the range of sites at which E2F family members can regulate transcription.

In the work presented here, E2F1, but not other E2F family members, was shown to bind to the carboxylesterase promoter. In previous studies, E2F1 was shown to specifically bind to and activate the transcription of thioether *S*-methyltransferase and hydroxysteroid sulfotransferase, which, like carboxylesterase, encode enzymes that metabolize various drugs and toxins (15). This E2F1-specific regulation of carboxylesterase and other detoxifying enzymes may suggest a new role for E2F1 in sensing and responding to various stresses, such as the presence of harmful compounds. Similarly to the carboxylesterase promoter, the thioether *S*-methyltransferase and hydroxysteroid sulfotransferase promoters also lack a consensus E2F site. Future studies are required to determine whether the mechanism by which E2F1 regulates the carboxylesterase promoter is

also used to regulate thioether *S*-methyltransferase and hydroxysteroid sulfotransferase expression.

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