Isolation of Two Novel cDNAs Whose Products Associate with the Amino Terminus of the E2F1 Transcription Factor†

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ABSTRACT: The amino terminus of the E2F1 transcription factor is a protein−protein interaction domain since it associates with cyclin A/cdk2. Here, the two-hybrid yeast screen was used to clone genes whose products associate with the amino terminus of E2F1. The amino-terminal 121 amino acids of E2F1 were fused to the Lex A binding domain while a partial length cDNA library from the embryo of a 12 day old mouse was fused to the VP16 activation domain. Following coexpression of these fusions in yeast, two novel genes were cloned that code for proteins that associate with E2F1. In an in vitro assay, these *E*2F1 *B*inding *P*roteins (EBP1 and EBP2) associate with residues 1−121 of E2F1 or with the full-length protein; however, they do not associate with its carboxy terminus (residues 88−437). When EBP1 or EBP2 were expressed in COS cells along with E2F1 and the target promoter DNA polymerase α, repression of transcription was observed. However, no repression of DNA polymerase α was seen if the cells expressed a nonassociating mutant E2F1 (residues 88−437), along with EBP1 or EBP2. Finally, expression of the EBP2 gene is up-regulated in growing NIH3T3 fibroblasts, relative to serum-starved cells. However, this up-regulation of EBP2 expression is not seen in fibroblasts constitutively expressing E2F1.

The E2F1 transcripton factor is part of a multiprotein family (Girling et al., 1993; Helin et al., 1992; Ivey-Hoyle et al., 1993; Huber et al., 1993; Kaelin et al., 1992; Lees et al., 1993; Ginsberg et al., 1994) and regulates the genes whose products are needed for initiation and completion of DNA synthesis (Nevins, 1992; Farnham et al., 1993; La Thangue, 1994). These genes include dihydrofolate reductase, DNA polymerase α, p34cdc2, thymidine kinase, and c-myc (Nevins, 1992; Farnham et al., 1993; La Thangue, 1994). E2F1, the first E2F family member to be cloned, is the best characterized to date. E2F1 binds the consensus DNA sequence TTTCGCGC within the promoters of the aforementioned genes, but does so optimally as part of a heterodimer with DP1 (Helin et al., 1993b). Additionally, E2F1 is known to physically interact with the retinoblastoma susceptibility gene product (pRb), MDM2, cyclin A, and cdk 2 (Helin et al., 1992; Kaelin et al., 1992; Cress et al., 1993; Krek et al., 1994; Xu et al., 1994; Martin et al., 1995). These interactions regulate the activity of E2F1. E2F1 contains what is thought to be a basic helix−loop−helix (bHLH) domain with an adjacent leucine zipper (Kaelin et al., 1992). The bHLH domain is required for DNA binding (Jordan et al., 1994); however, the structure of the basic domain of E2F1 appears unique among bHLH-containing proteins in that the center of the domain likely contains a turn instead of an α-helix (Jordan et al., 1994). The transcriptional activation domain of E2F1 resides at its carboxy terminus (Helin et al., 1992; Kaelin et al., 1992; Ginsberg et al., 1994). When pRb binds the C-terminus of E2F1, it blocks its ability to activate transcription (Helin et al., 1992, 1993a; Kaelin et al., 1992; Flemington et al., 1993).

Constitutive expression of E2F1 in NIH3T3 fibroblasts causes increased entry of the cells into S phase during serum starvation conditions, yet they are unable to complete S phase (Logan et al., 1994; Johnson et al., 1993; Krek et al., 1994). The primary cell cycle phase affected by constitutive expression of E2F1 appears to be G0, since this phase is dramatically shortened in serum-starved fibroblasts, while G1 phase is unaffected (Logan et al., 1996).

When a mutant E2F1 (an amino-terminal deletion termed E2F1d87) is constitutively expressed in fibroblasts, it causes the cells to undergo a number of phenotypic changes. The cells demonstrate a rounded morphology during culture in 10% calf serum, such that normal cytoskeletal structures as microfilaments, micrutubules, and vinculin containing focal contacts are not detectable (Logan et al., 1994). In addition, the cells have a lengthened S phase (Logan et al., 1995, 1996) and are more sensitive to S phase specific toxins (Logan et al., 1995). The E2F1d87 mutant lacks the cyclin A/cdk2 binding domain (Krek et al., 1994, Jordan and Hall unpublished observations). Thus, the lengthened S phase seen in cells expressing E2F1d87 is consistent with the loss of the cyclin A/cdk2 binding domain, resulting in partial arrest at the S phase checkpoint (Krek et al., 1995). The fact that cyclin A/cdk2 associates with the amino terminus of E2F1 led to the possibility that this region of the protein may be a site for protein−protein interaction. Here we explore this idea by analyzing the ability of nuclear factors to associate with the amino terminus of E2F1. Using a two-hybrid yeast screen, two novel cDNAs were cloned that code for proteins that associate specifically with the amino terminus of E2F1.

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MATERIALS AND METHODS

Cell Culture and Transfections. COS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. All transfection experiments were initiated on 50% confluent monolayer cultures as in Ausubel et al. (1987). The cells were glycerol-shocked 5−6 h after DNA addition. Plasmids (a total of 30 μg) were transfected by the calcium phosphate procedure (Moberg et al., 1992). The cells were glycerol-shocked 5−6 h after DNA addition. Extracts were generated approximately 48 h after the glycerol shock, and CAT activity was assayed from the soluble extracts. Equal amounts of protein were assayed for CAT activity by thin-layer chromatography. The transfections were performed multiple times. A plasmid containing the rous sarcoma virus long terminal repeat (RSV-LTR) driving the β-galactosidase gene (RSVb-gal) was included in all transfections of the CAT constructs so that the CAT activity could be normalized for differences in transfection efficiency.

Two-Hybrid Yeast Screen. (A) Strains and Media. The strains of *Saccharomyces cerevisiae* used in this study were those described by Vojtek et al. (1993). The reporter strain used was L40 (MATa,trp1,leu2,LYS2::lexA-HIS3,URA3::lexA-lacZ; Sternglanz, Weintraub, and Hollenburg unpublished data). Yeast cells were grown in rich media YPD_A (1% yeast extract, 2% bacto-peptone, 2% glucose, and 0.1 μg/mL adenine) or in synthetic media lacking amino acids for which the yeast cells are prototrophic due to the presence of a particular plasmid marker.

(B) Plasmids. pLexA-E2F(1−121) and pLexA-E2F1(60−121) contain the E2F1 coding sequence for amino acids 1−121 and 60−121, respectively. This sequence was cloned in-frame with the LexA coding sequence in plasmid pBTM116 (Fields & Song, 1989, Chien et al., 1991). The cDNA library was cloned into plasmid pVP16, a high-copy yeast vector which contains the coding region for the transcriptional activation domain of the herpes virus VP16 transactivator as well as the leu2 marker gene coding for leucine biosynthesis (Vojtek et al., 1993).

(C) Library Screen. L40 cells were transformed with pLexA-E2F1(1−121) by the lithium chloride method. The plasmid was maintained by prototrophy for the TRP1 marker. L40 containing pLexA-E2F1(1−121) was then prepared for electroporation of the library; 200 μg of library plasmid DNA was then electroporated into 50 μL aliquots of L40 containing pLexA-E2F1(1−121). Electroporated cells were plated onto media lacking Ura, Lys, Trp, Leu, and His. Growth on His-(−) plates indicates that an interaction between E2F1(1−121) and the protein was coded by the unknown cDNA. Colonies were then assayed for β-galactosidase activity to ensure that the interaction between E2F1(1−121) and the protein coded by the unknown cDNA was not specific for the His promoter. The pVP16 plasmid containing the unknown cDNA was then isolated by the "smash and grab" method (Vojtek et al., 1993), and the cDNA insert was sequenced.

In Vitro Transcription/Translation. In vitro transcription reactions were performed with plasmids containing the E2F1, E2F1d87, EBP1, and EBP2 cDNAs cloned into the *Eco*RI site of pBluescript II KS+. An initiator methionine sequence (ATG) was cloned into the *Hin*dIII site, just 5′ to the E2F1d87, EBP1, and EBP2 cDNAs. The constructs were linearized, and RNA was generated using purified T3 polymerase. Transcripts (315 −325 ng) were used to generate the E2F1, E2F1d87, EBP1, and EBP2 proteins using an in vitro translation reaction with a nuclease-treated rabbit reticulocyte lysate (35 μL, Promega) in a total reaction volume of 50 μL. [^{35}S]Methionine at 0.9 mCi/mL was also included in the reactions.

E2F1 Affinity Column Chromatography. E2F1(1−437), E2F1(88−437), E2F1(1−121), E2F1(241−437), EBP1, and EBP2 were cloned into pGEX-2T (Pharmacia) in-frame with the glutathione *S*-transferase gene (GST). Fusion proteins were produced as described in Jordan et al. (1994). Briefly, the proteins were induced with IPTG for 3 h, bacteria were lysed, and protein was separated from cellular debris by centrifugation. Using the bacterial extracts, affinity columns containing the various fusion proteins were then generated on glutathione−Sepharose (Pharmacia).

Radiolabeled E2F1, E2F1d87, EBP1, and EBP2 proteins, generated by in vitro transcription/translation as described above, were applied to the affinity columns containing the various GST-E2F1 constructs, GST-EBP1 or GST-EBP2. A column containing only GST was used as a negative control. The affinity columns were then washed with 5 bed volumes of NETN [150 mM NaCl, 20 mM Tris (pH 8), 1 mM EDTA, 0.5% NP40, 125 mM PMSF, 2 μg/mL pepstatin A, and 1 μg/mL leupeptin] and the bound proteins eluted by boiling the columns in SDS−PAGE sample buffer. The eluted proteins were then electrophoresed on a 10% SDS−polyacrylamide gel. The gels were dried and exposed to X-ray film.

COS Cell Expression. To express EBP1 and EBP2 in COS cells, they were first modified at their amino termini to contain an epitope tag (Met-Asp-Tyr-Lys-Asp-) recognized by a specific monoclonal antibody (M2, Kodak/IBI), followed by a nuclear localization sequence from the SV40 virus T antigen (Cys-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val). These modified cDNAs were then cloned into the COS cell expression vector pMT2 (Kaufman et al., 1989). Subconfluent proliferating cells were transfected with the pMT2-EBP1 or pMT2-EBP2 expression plasmids along with a DNA polymerase α−CAT promoter construct by the $CaPO_4$ method, as described above. Also included in the transfections was an E2F1 expression plasmid(pMT2-E2F1) or an E2F1d87 expression plasmid (pMT2-E2F1d87). A plasmid containing the rous sarcoma virus long terminal repeat (RSV-LTR) driving the β-galactosidase gene (RSVb-gal) was included in all transfections so that the CAT activity could be normalized for differences in transfection efficiency.

RESULTS

Two-Hybrid Screen Identifies Two Novel Proteins That Interact with the Amino Terminus of E2F1. Cyclin A/cdk2 has been shown to associate with the amino terminus of the E2F1 transcription factor. Krek et al. (1994) have mapped the region to residues 68−108, while Xu et al. (1994) have mapped the region to residues 79−124. Since these cyclin A/cdk2 specifically associate with the amino terminus of E2F1, the possibility exists that other cellular factors may also bind to this region. Preliminary in vitro experiments using E2F1 affinity columns in protein association assays indicated that a number of nuclear proteins bind to the amino terminus of E2F1 (Jordan and Hall, unpublished observa-

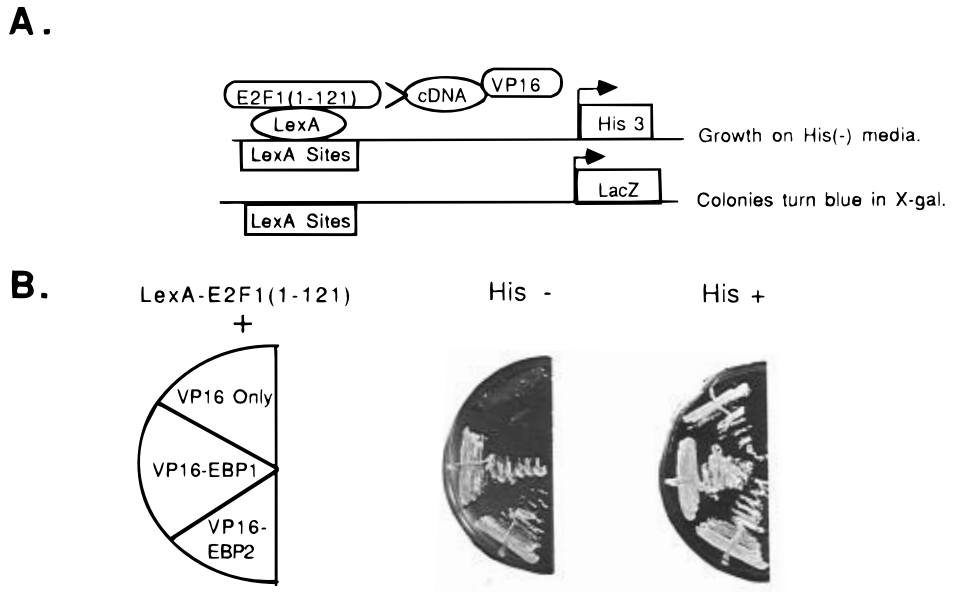

FIGURE 1: Two-hybrid yeast screen identifies two genes that code for E2F1-interacting proteins. (A) Shown is an outline of the two-hybrid screen. A partial length cDNA library from 8.5−10.5 day old mouse embryos was linked in-frame with the transcriptional activation domain of the herpes viral protein VP16. E2F1(1−121) is linked in-frame with the bacterial DNA binding protein LexA. Target promoters containing multiple LexA sites drive expression of the His 3 and β-galactosidase genes, contained within the genome of *Saccharomyces cerevisiae* strain L40. Interaction of a gene product with E2F1(1−121) will promote transcription of His 3 and allow for growth on His minus media. (B) Interaction of gene products with the amino-terminal residues 1−121 of E2F1. Yeast transformed with the cDNA library expression vector and the LexA-E2F1(1−121) expression vector were plated on media lacking histidine. Colonies that grew were selected; the plasmids containing the unknown cDNA were isolated and used to retransform yeast. The yeast were streaked onto plates containing (His +) or lacking (His −) histidine. Coexpression of LexA-E2F1(1−121) and VP16-EBP1 or VP16-EBP2 allows growth of yeast on plates lacking histidine. As a negative control, the yeast were transformed with the LexA-E2F1(1−121) expression plasimd and pVP16 (library vector only).

tions). To directly clone the cDNAs for these proteins that interact with the amino terminus of E2F1, a two-hybrid screen was performed in the yeast *Saccharomyces cerevisieae* [as in Vojtek et al. (1993), Fields and Song (1989), and Chien et al. (1991)]. In one yeast expression plasmid, the amino-terminal 121 residues of E2F1 were linked to the DNA binding domain of the bacterial protein LexA (Vojtek et al., 1993). In a second yeast expression plasmid, a partial length cDNA library from 8.5−10.5 day old mouse embryos was cloned adjacent to the acidic domain of the Herpes virus transcriptional activator VP16 (Vojtek et al., 1993). Targets for the interaction are the genes encoding histidine biosynthesis (His 3) and β-galactosidase, both of which have promoters containing LexA sites. This is diagrammed in Figure 1A. Interaction between the amino terminus of E2F1 and the protein encoded by the cDNA will lead to increased expression of His3 and β-gal, resulting in selection for growth on histidine minus media and for blue color selection in the presence of X-gal.

To perform the screen, yeast (strain L40) were transformed with the LexA-E2F1(1−121) expressing plasmid and the cDNA Library-VP16 expressing plasmid. The yeast were plated on His minus media. A total of 500 000 transformants were screened. Thirty colonies were identified that grew on histidine minus media; the plasmids containing the unknown cDNAs were isolated and used to retransform yeast to ensure histidine prototrophy. Most of the rescued cDNAs failed to promote histidine prototrophy upon retransformation. However, two cDNAs did allow growth on histidine minus media and were subjected to further analysis. The results of this selection are shown in Figure 1B. It is clear that the genes encoding the two E2F1 binding proteins (EBP1 and EBP2) provide a stable interaction with E2F1(1−121) in vivo, enabling the yeast to grow on His minus media. When analyzed for their ability to activate β-galactosidase, it was found that the colonies of yeast expressing EBP1 and EBP2 turned blue in the presence of X-gal, while the yeast containing a control plasmid did not. From these data, it is clear that the amino terminus of E2F1 interacts with EBP1 and EBP2, leading to increased expression of the His 3 and β-galactosidase genes. As a control, we determined if VP16-EBP1 and VP16-EBP2 would interact with a nonspecific protein such as nuclear lamin (i.e., LexA−lamin fusion). Yeast transformed with a LexA−lamin fusion protein and either VP16-EBP1 or VP16-EBP2 did not promote growth on histidine minus media, indicating that the interaction is not nonspecific (data not shown).

EBP1 and EBP2 Are Novel cDNAs. The DNA and corresponding predicted amino acid sequences for EBP1 and EBP2 are shown in Figure 2A,B. The amino acid sequence for EBP1 and EBP2 is the peptide sequence that is in-frame with the LexA amino acid sequence, resulting in the formation of a functional fusion protein. A search of GENBANK and SwissProt databases, with both the DNA and amino acid sequences of EBP1 and EBP2, revealed no identities with any other genes or proteins in the databases including known partners of E2F1 such as DP1. Also, these proteins did not appear to contain any recognizable domains such as leucine heptad repeats, basic helix−loop−helix domains, etc. that would be involved in protein−protein interactions.

In Vitro Association of EBP1 and EBP2 with E2F1. To determine if EBP1 and EBP2 associate with the amino terminus of E2F1 in vitro, column assays were performed. Residues 1−121, 1−437, 88−437, and 241−437 of E2F1 were fused to glutathione *S*-transferase (GST). The fusion

A **EBP1.**

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           10          20          30          40          50
            *           *           *           *           *
CGG CCG CTC ACA CCT AAC CCC TCC AGA GGA TCG TCC TTG CCT AGA ACC CTC TCC
Arg Pro Leu Thr Pro Asn Pro Ser Arg Gly Ser Ser Leu Pro Arg Thr Leu Ser>

      60          70          80          90          100
       *           *           *           *           *
AAA GAG TCC AAG CTG TAT GCT AAA GGA AAA GGA AGT GGA GGA GTA AAA ACT GCC
Lys Glu Ser Lys Leu Tyr Ala Lys Gly Lys Gly Ser Gly Gly Val Lys Thr Ala>

110         120         130         140         150         160
  *           *           *           *           *           *
AAG CTC TAT GCC TGG GTA GCA CTG CAG TCA TTA CCA GAA GAA ATG GTT ATT AGT
Lys Leu Tyr Ala Trp Val Ala Leu Gln Ser Leu Pro Glu Glu Met Val Ile Ser>

       170         180         190         200         210
         *           *           *           *           *
CCT TGT CTA TTG GAC TTT CTT GAA AAG GCT CTG GAA ACT ATC CCA ATT ACA CCA
Pro Cys Leu Leu Asp Phe Leu Glu Lys Ala Leu Glu Thr Ile Pro Ile Thr Pro>

   220         230         240         250         260         270
    *           *           *           *           *           *
ATC GAA AGG AAT TAT ACA GCA GTC AGC TCA CAA GAT GAA GAT ATG GGA CAT TTT
Ile Glu Arg Asn Tyr Thr Ala Val Ser Ser Gln Asp Glu Asp Met Gly His Phe>

           280         290         300         310         320
            *           *           *           *           *
GAC ATC CCA GAT CCT ATG GAA GAA TCA ACA ACA TCC CTA GGG TCA TCT TCA ACA
Asp Ile Pro Asp Pro Met Glu Glu Ser Thr Thr Ser Leu Gly Ser Ser Ser Thr>

     330         340         350         360         370
      *           *           *           *           *
CCT GCA TAC TCT TCC TTC CCT GTA GAC GTT GTG GTT TAT GTG CGA GTT CAG CCC
Pro Ala Tyr Ser Ser Phe Pro Val Asp Val Val Val Tyr Val Arg Val Gln Pro>

380         390         400         410         420         430
  *           *           *           *           *           *
TCA CAG ATC AAA TTT AGC TGT TTG CCA GTA TCA AGA GTA GAA TGC ATG CTG AAG
Ser Gln Ile Lys Phe Ser Cys Leu Pro Val Ser Arg Val Glu Cys Met Leu Lys>

       440         450         460         470         480
         *           *           *           *           *
CTA CCA TCT CTG GAC TTG GTA TTT TCT TCA AAC CGA GGA GAA CTG GAA ACT TTG
Leu Pro Ser Leu Asp Leu Val Phe Ser Ser Asn Arg Gly Glu Leu Glu Thr Leu>

   490         500         510
    *           *           *
GGG ACT ACA TAC CCT GCA GAG ACC GTG TCC CCT
Gly Thr Thr Tyr Pro Ala Glu Thr Val Ser Pro
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B **EBP2.**

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           10          20          30          40          50
            *           *           *           *           *
CTC ACC CCC CAG CGA CAG TTT CAC GAC AAC GCC AAT GGG GGT CAG AAT GGC ACA
Leu Thr Pro Gln Arg Gln Phe His Asp Asn Ala Asn Gly Gly Gln Asn Gly Thr>

      60          70          80          90          100
       *           *           *           *           *
GTG CAG GAG ATC ATG ATC CCT GCT GGC AAG GCT GGC CTG GTC ATT GGT AAA GGT
Val Gln Glu Ile Met Ile Pro Ala Gly Lys Ala Gly Leu Val Ile Gly Lys Gly>

110         120         130         140         150         160
  *           *           *           *           *           *
GGA GAG ACC ATC AAG CAG CTG CAG GAG CGG GCT GGG GTG AAG ATG ATT TTA ATT
Gly Glu Thr Ile Lys Gln Leu Gln Glu Arg Ala Gly Val Lys Met Ile Leu Ile>

       170         180         190         200         210
         *           *           *           *           *
CAG GAT GGC TCC CAG AAC ACA AAT GTG GAC AAG CCA CTG CGG ATT ATC GGG GAC
Gln Asp Gly Ser Gln Asn Thr Asn Val Asp Lys Pro Leu Arg Ile Ile Gly Asp>

   220         230         240         250         260         270
    *           *           *           *           *           *
CCA TAC AAA GTA CAG CAA GCC TGT GAG ATG GTG ATG GAC ATC CTG CGA GAA CGT
Pro Tyr Lys Val Gln Gln Ala Cys Glu Met Val Met Asp Ile Leu Arg Glu Arg>

           280         290         300         310         320
            *           *           *           *           *
GAC CAA GGT GGC TTT GGG GAC CGC AAT GAA TAC GGA TCT CGA GTT GGT GGA GGC
Asp Gln Gly Gly Phe Gly Asp Arg Asn Glu Tyr Gly Ser Arg Val Gly Gly Gly>

     330         340         350         360         370
      *           *           *           *           *
ATT GAT GTG CCT GTA CCC AGG CAT TCA GTT GTG AGC AAT CTT CTC AGG GCC GGT
Ile Asp Val Pro Val Pro Arg His Ser Val Val Ser Asn Leu Leu Arg Ala Gly>

380         390         400         410         420         430
  *           *           *           *           *           *
CCC ATC ATC TTG CTT GAA CTG GAT GCG CAC ACC GGC ATC ATT CTG GAT CTT CTT
Pro Ile Ile Leu Leu Glu Leu Asp Ala His Thr Gly Ile Ile Leu Asp Leu Leu>

       440         450         460
         *           *           *
GAT CAT CTC TCC ACT CCG GCA ATG ACT ACA CCT
Asp His Leu Ser Thr Pro Ala Met Thr Thr Pro
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FIGURE 2: DNA sequence and predicted open-reading frame of EBP1 and EBP2. DNA sequence analysis is shown with the corresponding predicted amino-acid sequence of the two E2F1 binding proteins, EBP1 (A) and EBP2 (B). Search of both GENBANK and Swiss-Prot databases revealed no homology to any known genes or proteins, respectively. Additionally, no known motifs, such as leucine zipper, helix−loop−helix, or nuclear localization sequence, were present in the protein sequence.

proteins were produced in bacteria and used to make affinity columns on glutathione−Sepharose. The EBP1 and EBP2 cDNAs were next modified such that a translation initiator methionine sequence, along with a Kozak consensus sequence, was placed at their very 5′ end. In this way, the EBP1 and EBP2 proteins could be radiolabeled ([^{35}S]Met) by coupled in vitro transcription/translation (the latter by a rabbit reticulocyte lysate). The radiolabeled EBP1 and EBP2 proteins were individually applied to affinity columns containing the various GST-E2F1 fusion proteins. The columns were washed extensively, and then the bound proteins were eluted by boiling the columns in SDS−PAGE sample buffer. As shown in Figure 3A, EBP1 associates equally well with residues 1−121 and 1−437 but does not associate with 88−437 or 241−437 or with GST alone. EBP2, on the other hand (Figure 3B), associates with residues 1−437 and 1−121, but does not associate with residues 88−437 or 241−437 or with GST alone. At present, we do not know the identity of the two bands in Figure 3B that migrate faster than the EBP2 protein. It is likely that they are proteolytic breakdown fragments of EBP2. However, since they bind GST, it is likely that their association with residues 1−121 and 1−437 of E2F1 is nonspecific. Figure 3C shows a Coomassie blue stained gel containing the fusion proteins, indicating that roughly equal amounts of fusion proteins were present on the columns. To rule out the possibility that EBP1 and EBP2 do not bind GST-E2F1(88−437), because of a problem with protein folding, for example, the DP1 protein was radiolabeled by in vitro transcription/translation and passed over the GST fusion columns as shown in Figure 3D. From the data, it is clear that DP1 binds to GST-E2F1-(88−437) as efficiently as to GST-E2F1(1−437), indicating that the amino-terminal mutant retains the proper structure enabling it to dimerize with DP1. Thus, these data indicate that in vitro, EBP1 and EBP2 specifically associate with the amino terminus of E2F1 as opposed its carboxy terminus.

To confirm the above observations, we next performed the converse experiment. EBP1 and EBP2 were fused in-frame to the carboxy terminus of GST. The fusion proteins were produced in bacteria and used to generate affinity columns on glutathione−sepharose. E2F1, residues 1−437 and residues 88−437, were then radiolabeled ([^{35}S]Met) by coupled in vitro transcription/translation as described above for EBP1 and EBP2. The labeled proteins were then individually applied to the affinity columns. As shown in Figure 4A, it is clear that only E2F1(1−437) associates with the EBP1 and EBP2 affinity columns, while E2F1(88−437) does not. These data are consistent with the data in Figure 3. Thus, EBP1 and EBP2 associate with the amino terminus of E2F1 but not with the remainder of the protein.

Transcripts for EBP2 Show Varying Levels of Expression in Transformed and Nontransformed Mouse Cell Lines. The levels of expression of the EBP1 and EBP2 genes in various cell lines were next assessed to better understand how expression of these genes is regulated. The EBP1 and EBP2 cDNAs were therefore ^{32}P-labeled and used to probe a Northern blot containing RNAs from a number of transformed and nontransformed cell lines (both human and mouse). After multiple attempts, EBP1 could not be detected in the blot from multiple cell lines. It is possible that its level of expression is very low or that it is restricted to a specific period in development. The EBP2 gene was found to be expressed in a number of distinct cell lines (Figure 5A), producing a transcript of approximately 4 kb in size; however, the level of expression varied significantly. The highest level of expression was found in nontransformed growing NIH3T3 fibroblasts and NIH3T3 fibroblasts transformed by a Grb2-associated docking protein (Pilar 9 cells; Holgado-Madruga et al., 1996). Intermediate levels of expression were observed in A431 cells (epidermal carci-

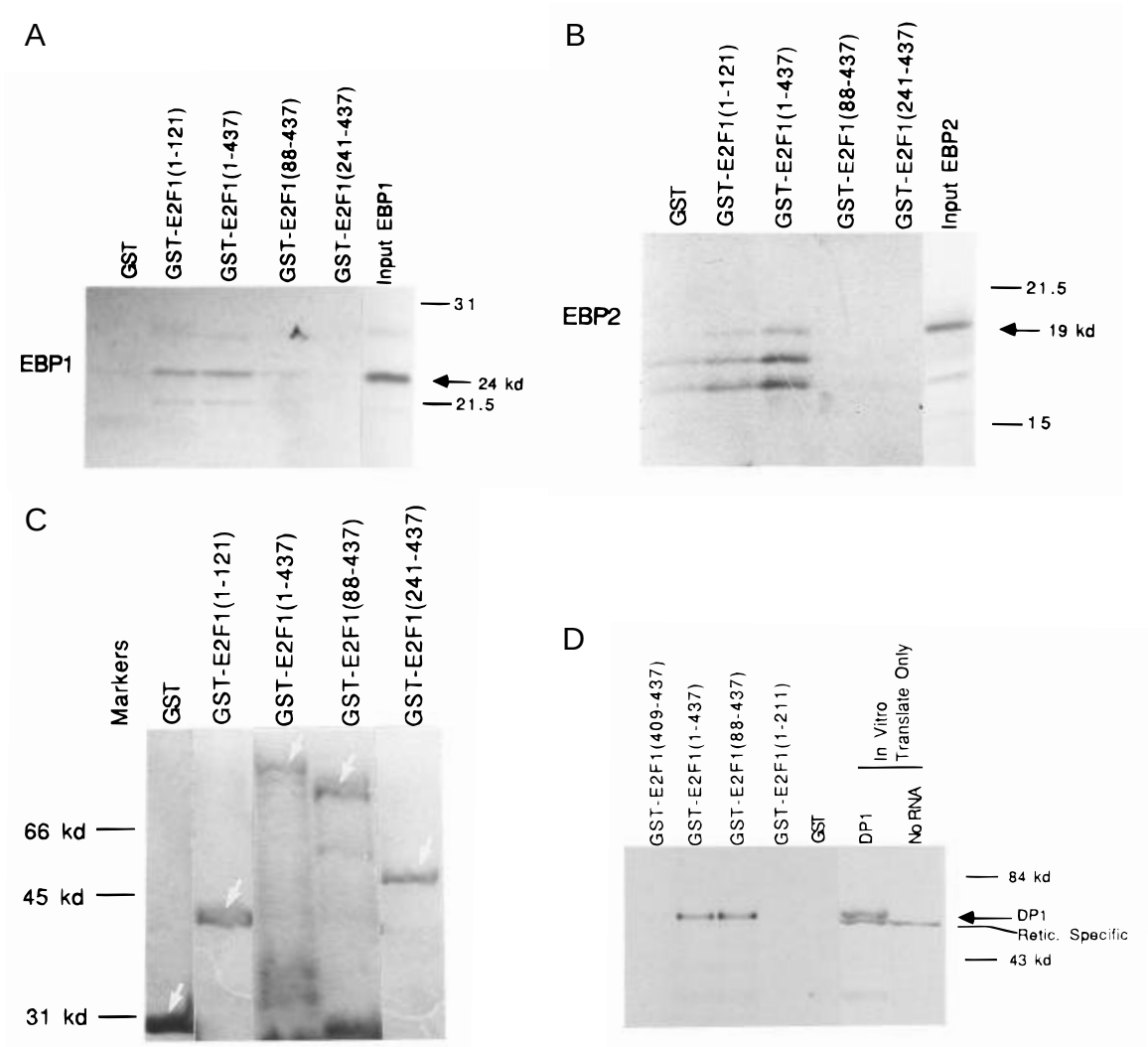


FIGURE 3: EBP1 and EBP2 associate with column-immobilized E2F1. EBP1 and EBP2 were modified so that they contained a translation initiator methionine at their amino termini. The genes were then transcribed in vitro, and the RNA was used to make protein by in vitro translation in a rabbit reticulocyte lysate (containing [^{35}S]Met). The labeled EBP1 (A) and EBP2 (B) proteins were passed over columns containing the indictated portions of E2F1 fused to glutathione *S*-transferase (GST), the columns were washed extensively, and the bound material was eluted and fractionated by SDS−PAGE. A column containing just GST served as a negative control. Shown are autoradiographs of the gels. Also included in the gels are the input-translated EBP1 and EBP2 proteins (indicated by the arrows). The numbers to the right indicate the sizes of the marker bands, in kilodaltons. Panel C shows the Coomassie blue stained gel containing the various GST-fusion proteins, indicating relatively equal levels of fusion protein on the columns. (D) The DP1 gene was transcribed and translated in vitro using a rabbit reticulocyte lysate (containing [^{35}S]Met). The labeled DP1 protein was passed over columns containing the indictated portions of E2F1 fused to GST, the columns were washed extensively, and the bound material was eluted and fractionated by SDS−PAGE. A column containing just GST served as a negative control. Shown is an autoradiograph of the gels. Also included in the gels are the input-translated DP1 protein (indicated by the arrow). The numbers to the right indicate the sizes of the marker bands, in kilodaltons. The band labeled "Retic. Specific" is an unknown protein labeled in the reticulocyte extract from endogenous RNA present in the extract (likely globin).

noma), COS cells (transformed monkey kidney), and HeLa cells (cervical carcinoma). Low levels of expression were seen in transformed T-cells (J32), glioblastoma cells (U87MG), and transformed B-cells (Daudi). These data indicate that expression of the EBP2 gene may be restricted to specific cell types. The highest level was seen in proliferating transformed fibroblasts. To extend these experiments RNAs derived from 3 month old mouse tissues were subject to Northern analysis with both EBP1 and EBP2 probes. EBP1 was not detectable on the blots; however, as shown in Figure 5B, EBP2 was present in all tissues examined. This may be due to the fact that the mice were young and many of the tissues had substantial amounts of cell proliferation. Interestingly, the transcript size present in the RNA from mouse skin was smaller.

Since EBP2 transcript levels were highest in growing fibroblasts (transformed and nontransformed), an experiment was performed to the level of expression during quiescence. RNA was isolated from nontransformed NIH3T3 fibroblasts that were growing or were made quiescent by culture for 48 h in 0.5% calf serum. The RNA was blotted and probed with radiolabeled EBP2 cDNA, as described above. As seen in Figure 6A, EBP2 gene expression is significantly down-regulated during conditions of serum withdrawal, as the cells enter into a G0 phase. Thus, for nontransformed fibroblasts in tissue culture, EBP2 gene expression is induced in growing cells.

Also examined were the EBP2 transcript levels in NIH3T3 fibroblasts constitutively expressing E2F1 (Logan et al., 1994). Interestingly, as seen in Figure 6A, EBP2 mRNA levels are not up-regulated in the growing "E2F1" cell line, cultured in 10% serum. From these data, it appears that E2F1 affects EBP2 gene expression. Figure 6B shows flow cytometry data from the cell in Figure 6A, indicating the

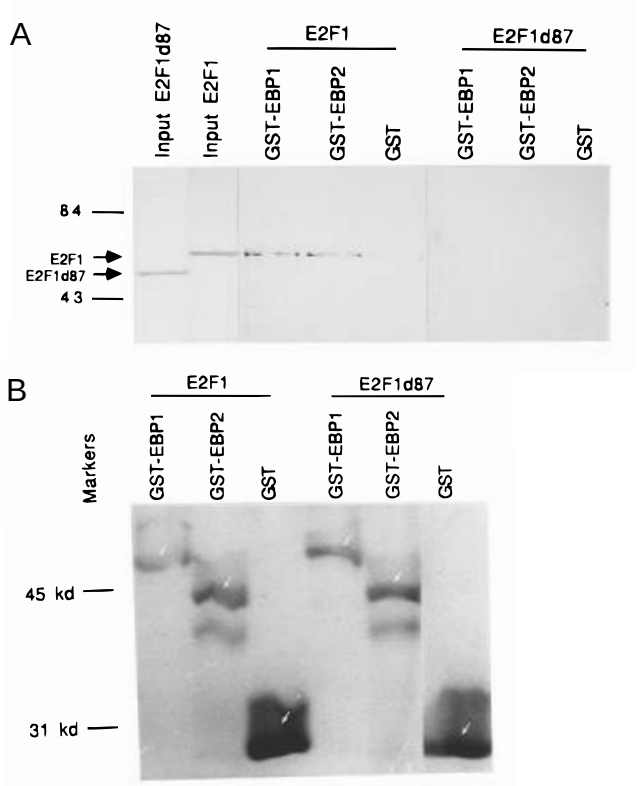


FIGURE 4: Full-length E2F1 but not E2F1(88−437) associates with column-immobilized EBP1 and EBP2. (A) E2F1(1−437) and E2F1-(88−437) were transcribed and translated in vitro (in reactions containing [^{35}S]Met), as in Figure 3. The labeled proteins were then passed over columns containing either EBP1 or EBP2 fused to GST (GST alone served as a control). The columns were washed extensively and then the bound proteins eluted and fractionated by SDS−PAGE. Shown is an autoradiograph of the gel. Also included in the gel are the input translated E2F1(1−437) and E2F1(88−437) proteins (indicated by the arrows). The numbers to the left indicate the sizes of the marker bands, in kilodaltons. (B) Shown is the Coomassie blue stained gel containing the the various GST-fusion proteins indicating relatively equal levels of fusion protein on the columns.

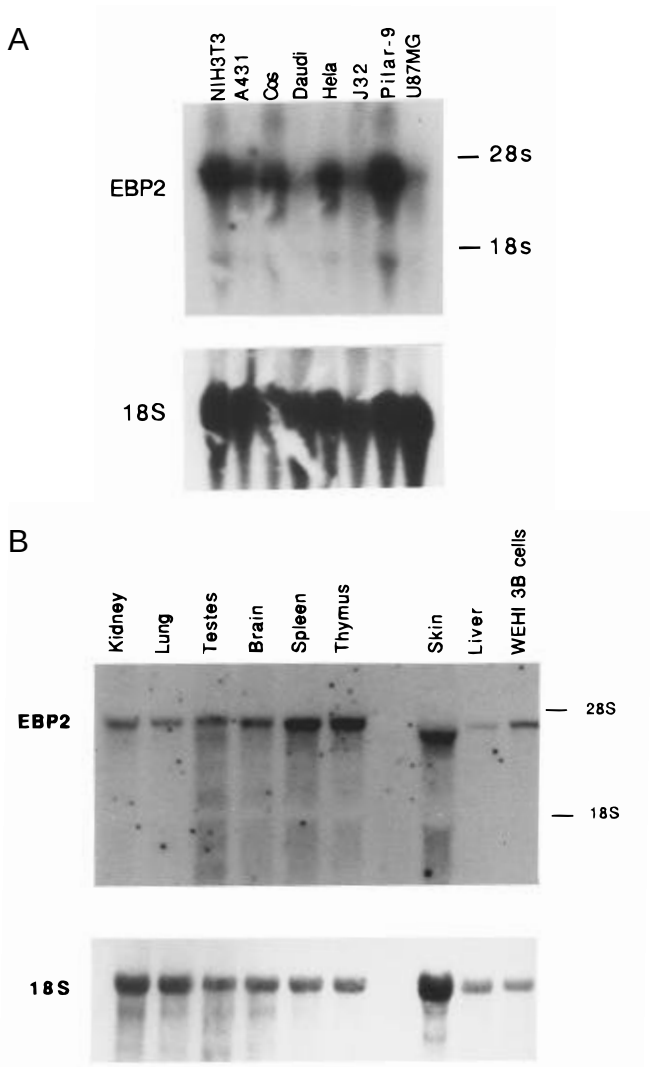


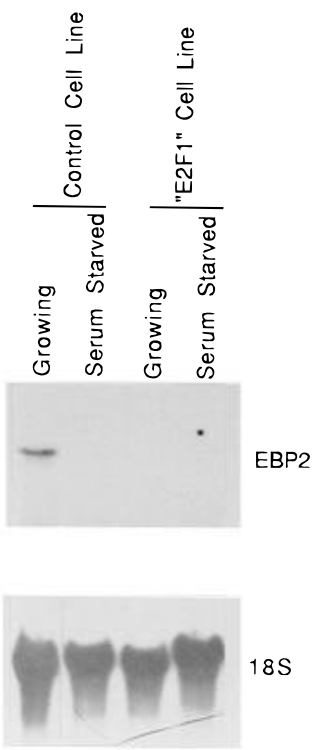
FIGURE 5: EBP2 gene expression shows variation between a variety of transformed and nontransformed cell lines. (A) The EBP2 cDNA was gel-purified, ^{32}P-labeled, and used to probe Northern blots containing RNA (20 μg/lane) from transformed and nontransformed human and mouse cell lines. The arrows point to the EBP2 message, migrating just ahead of the 28S rRNA. The cell lines are NIH3T3 fibroblasts (murine embryonic), A431 (epidermal carcinoma), COS (transformed monkey kidney), Daudi (transformed B-cells), HeLa (human cervical carcinoma), J32 (transformed T-cells), Pilar-9 (transformed mouse fibroblasts), and U87MG (human glioblastoma). As a control, the blot was probed with a radiolabeled 18S rRNA probe, to show equal levels of RNA per lane (lower portion). (B) The EBP2 cDNA was labeled as in (A) and used to probe a blot containing RNA (20 μg) from various tissues of a 3-month old mouse. As a control, the blot was probed with a radiolabeled 18S rRNA probe, to show equal levels of RNA per lane (lower portion).

distribution of cells through the cell cycle. From this, it is evident that "growing" cells are more evenly distributed throughout the cell cycle while the serum-starved cells are primarily arrested in the G1 phase. As reported earlier (Logan et al., 1994, 1996), during serum starvation, an increased number of the E2F1-expressing cells pass into S phase yet do not enter G2/M phase (Logan et al., 1996, and Figure 6B).

Ectopic Expression of the EBP1 and EBP2 Genes in COS Cells Leads to Reduced Expression of the E2F Target Promoter DNA Polymerase α. Since EBP1 and EBP2 associate with E2F1, it is important to know if their association affects the ability of E2F1 to activate transcription. To determine this, the EBP1 and EBP2 cDNAs were cloned into a mammalian expression vector. The EBP2 cDNA is 500 bp while its transcript is nearly 4 kb, indicating that it is not a full-length cDNA. It is likely that the EBP1 cDNA is also not full length; the cDNA library was intentionally made partial length to enhance interactions between protein domains (Vojtek et al., 1993). Upon examination of the open reading frames of EBP1 and EBP2, it is evident that they do not contain a nuclear localization sequence. They were therefore first modified to include both an epitope tag for efficient detection and the nuclear localization sequence (NLS) from SV40 virus T antigen: Cys-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val. Both the tag and NLS were engineered into the amino termini of the genes (e.g., tag-NLS-EBP1). The modified cDNAs were then cloned into the pMT2 expression vector (Kaufman et al., 1989) for high level of expression in COS cells.

To determine if the modified proteins were transported efficiently to the nucleus, indirect immunofluorescence was first performed. COS cells were seeded onto glass coverslips and then individually transiently transfected with the expression plasmids. Forty-eight hours after the transfection, the cells were fixed in paraformaldehyde and processed for indirect immunofluorescence using the M2 antibody directed against the epitope tag as the primary antibody (Pyrc et al.,

A



B

Flow Cytometry

	G1	S	G2/M
Subconfluent/Growing Cells			
E2F1 Cell Line	48%	36%	16%
Control Cells	42%	44%	14%
Serum Starved Cells			
E2F1 Cell Line	70%	25%	5%
Control Cells	92%	4%	4%

FIGURE 6: EBP2 expression is growth-regulated in normal NIH3T3 fibroblasts but not in fibroblasts constitutively expressing E2F1. NIH3T3 cells alone (Control Cell Line) and NIH3T3 cells constitutively expressing the E2F1 transcription factor (E2F1 Cell Line) were analyzed for growth-regulated EBP2 expression. (A) RNA (20 μg/lane) isolated from subconfluent and proliferating NIH3T3 cells (Growing) or cultured for 48 h in 0.5% calf serum (Quiescent) was blotted and probed with radiolabeled EBP2 cDNA. RNA (20 μg/lane) from NIH3T3 cells constitutively expressing E2F1 was analyzed in the same fashion. As a control, the blot was probed with a radiolabeled 18S rRNA probe, to show equal levels of RNA per lane (lower portion). (B) Flow cytometry of the cells cultured in (A). Aliquots of the parental NIH3T3 cells ("Control Cells") and the NIH3T3 cells constitutively expressing E2F1 ("E2F1 Cell Line") from (A) above were processed for flow cytometry. Shown is the percent cells in each cell cycle phase.

1994). The secondary antibody was conjugated to fluorescein. From the micrographs in Figure 7, it is clear that the proteins are efficiently targeted to the nucleus.

Next, cotransfection assays were performed in COS cells. An E2F1 target promoter, DNA polymerase α−CAT, was cotransfected along with the individual pMT2-EBP constructs. Included in the transfections was an E2F1-expressing plasmid (pMT2-E2F1) allowing a high level of expression from the DNA polymerase α promoter. Forty-eight hours after the transfection, extracts were generated and assayed for CAT activity. When transfected into COS cells, both EBP1 and EBP2 expression decreased the level of transcription from the DNA polymerase α promoter, when compared to the control with pMT2 alone, as shown in Table 2. This repressive effect was found to be dose-dependent (Table 1), such that at 50 ng of effector plasmid the repression was

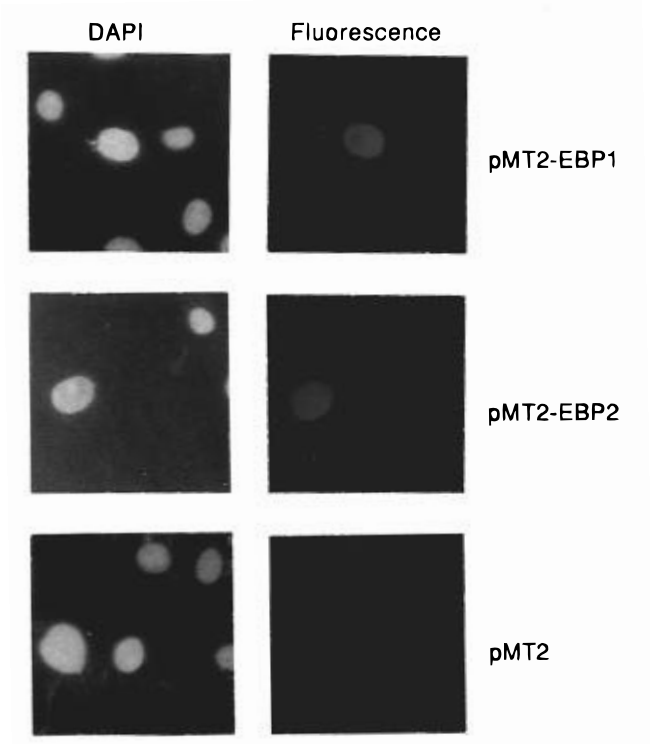


FIGURE 7: EBP1 and EBP2 proteins are efficiently targeted to the nucleus when modified to contain a nuclear localization sequence. The EBP1 and EBP2 reading frames do not show any consensus nuclear localization sequence. To ensure that they are efficiently targeted to the nucleus, a nuclear localization sequence derived from SV40 virus T antigen (Cys-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val) was engineered into their cDNAs, at the amino terminus. An epitope tag (Met-Asp-Tyr-Lys-Asp) was also engineered into the amino terminus. The epitope tag is recognized by the M2 monoclonal antibody (Kodak/IBI). The modified EBP1 and EBP2 cDNAs were cloned into the COS cell expression vector pMT2 and then transfected into COS cells grown on coverslips. Forty-eight hours after the transfection, the coverslips were processed for indirect immunofluorescence using the M2 antibody as the primary antibody and a fluorescein-conjugated anti-mouse antibody as a secondary antibody. The figure shows the nuclear fluorescence of the EBP1 (A, top), EBP2 (B, middle), and vector control (C, bottom) transfected cells. Shown on the left is DAPI staining of the DNA.

Table 1: Repression of E2F1-Dependent Transcription from the DNA Polymerase α Promoter by EBP1 and EBP2[a]

promoter and affecters	relative CAT activity (%, ±SD)
DNA pol α CAT + pMT2	30 ± 10
DNA pol α CAT + E2F1 + pMT2 (50 ng)	100.0 ± 19
DNA pol α CAT + E2F1 + pMT2-EBP1 (50 ng)	38.8 ± 15
DNA pol α CAT + E2F1 + pMT2-EBP2 (50 ng)	39.9 ± 8
DNA pol α CAT + E2F1 + pMT2 (500 ng)	97.0 ± 20
DNA pol α CAT + E2F1 + pMT2-EBP1 (500 ng)	18 ± 8
DNA pol α CAT + E2F1 + pMT2-EBP2 (500 ng)	17 ± 12

[a] COS cells were transfected with DNA polymerase α−CAT (2 μg) and pCMV-E2F1 (50 ng). The pMT2 and pMT2-EBP constructs were transfected at the indicated levels. The total level of DNA in the transfection was kept constant at 20 μg. After 48 h, the cells were harvested, and the resulting CAT activity was determined. A pSV2-β-galactosidase expression plasmid (0.5 μg) was also included in all the transfections as a control to normalize for differences in transfection efficiency. Shown is the relative CAT activity ± the standard deviation.

approximately 3-fold while at 500 ng of effector plasmid the repression was approximately 5-fold. Use of a mutant DNA polymerase α promoter lacking the E2F site at position −121 (Sala et al., 1994) shows no transactivation by added E2F1 and no repression by added EBP1 or EBP2 in the

Table 2: EBP1 and EBP2 Do Not Have a Repressive Effect on E2F1d87-Dependent Transcription from the DNA Polymerase α Promoter[a]

promoter and affecters	relative CAT activity (%, ±SD)
DNA pol α CAT + pMT2	34 ± 3
DNA pol α CAT + E2F1d87 + pMT2 (50 ng)	100 ± 21
DNA pol α CAT + E2F1d87 + pMT2-EBP1 (50 ng)	97 ± 14
DNA pol α CAT + E2F1d87 + pMT2-EBP2 (50 ng)	140 ± 8
DNA pol α CAT + E2F1d87 + pMT2 (500 ng)	98 ± 16
DNA pol α CAT + E2F1d87 + pMT2-EBP1 (500 ng)	120 ± 28
DNA pol α CAT + E2F1d87 + pMT2-EBP2 (500 ng)	110 ± 15

[a] COS cells were transfected with DNA polymerase α−CAT (2 μg) and pCMV-E2F1d87 (50 ng). The pMT2 and pMT2-EBP constructs were transfected at the indicated levels. The total level of DNA in the transfection was kept constant at 20 μg. After 48 h, the cells were harvested, and the resulting CAT activity was determined. A pSV2-β-galactosidase expression plasmid (0.5 μg) was also included in all the transfections as a control to normalize for differences in transfection efficiency. Shown is the relative CAT activity ± the standard deviation.

cotransfection assay (data not shown), indicating that repression occurs through the E2F site within the promoter.

As shown above in Figures 3 and 4, EBP1 and EBP2 do not associate with the mutant E2F1d87. Therefore, EBP1 and EBP2 should not affect the ability of E2F1d87 to transactivate the DNA polymerase α promoter. When cells were transfected with pMT2-E2F1d87, transactivation of the DNA polymerase α promoter was evident, as shown in Table 2. However, this transactivation was not repressed by EBP1 and EBP2 expression (Table 2). Taken together, the data of Tables 1 and 2 indicate that EBP1 and EBP2 are able to functionally alter the activity of E2F1 in vivo but that this alteraltion requires the amino terminus of E2F1.

DISCUSSION

The E2F1 transcription factor is composed of distinct domains. The carboxy terminus is the site for transcriptional activation and also contains two regions for the binding of the retinoblastoma protein (pRb) and MDM2 (Helin et al., 1992; Kaelin et al., 1992; Cress et al., 1993; Krek et al., 1994; Xu et al., 1994; Martin et al., 1995). Near the central portion of E2F1 is the DNA binding domain and the site for dimerization with the DP1 factor (Helin et al., 1993b). Located on the amino-terminal side of the DNA binding domain is the site for cyclin A/cdk2 binding (Krek et al., 1994; Xu et al., 1994). Finally, the very amino-terminal portion of the protein is a region rich in proline and alanine residues (Helin et al., 1992; Kaelin et al., 1992). Here we have analyzed protein association with the amino terminus of E2F1, a region that encompasses the cyclin A/cdk2 binding domain. Based on preliminary biochemical evidence that a number of nuclear proteins from murine fibroblasts associate with the amino terminus of E2F1, a two-hybrid yeast screen was employed to rapidly identify cDNAs that code for such proteins. Using this technique, two novel cDNAs were isolated; their DNA and predicted protein sequence does not correspond with any known genes in the GENBANK or SwissProt databases, respectively. Further, the open reading frames of EBP1 and EBP2 contain no recognizable motifs such as a leucine zipper, helix−loop−helix, or ankyrin repeats, that would comprise known interaction domains, indicating that the domains appear to be novel.

Analysis of EBP1 and EBP2 transcript levels revealed that the two genes show different levels of expression. EBP1 transcripts were not detectable in multitissue Northern blots or in the RNA from diverse cell lines, both transformed and nontransformed. It is possible that it is expressed at very low levels or that its expression is restricted to a defined window of time during mouse development (the cDNA library was generated from 8.5−10.5 day old mouse embryos). Current efforts are underway to distinguish these possibilities.

Expression of the EBP2 gene was evident in a variety of cell lines. The RNA was approximately 4 kb in size and expressed at highest levels in growing mouse fibroblasts that are either transformed or nontransformed. Expression was lower in COS, HeLa, T-cell, and B-cell lines. Of particular interest was the finding that EBP2 expression was growth-regulated in fibroblasts. Its transcript levels were increased about 10-fold in growing cells relative to serum-starved cells. Further, this increase was not seen in growing fibroblasts that constitutively express E2F1; transcript levels were very low independent of whether the cells were growing or serum-starved. Thus, E2F1 appears to affect the expression of EBP2. At this time, it is not known whether this effect is direct or indirect.

To get a better understanding of the function of EBP1 and EBP2, experiments were performed to determine if the two genes could affect E2F1-mediated transcriptional transactivation. DNA polymerase α was chosen as an E2F1 target promoter. In the presence of increased E2F1 expression, this promoter is transactivated (Table 1). In the presence of EBP1 or EBP2, transcription from the DNA polymerase α promoter was significantly repressed. Importantly, repression did not occur when E2F1(88−437) was used to transactivate the DNA polymerase α promoter, due to the fact that EBP1 and EBP2 do not associate with E2F1(88−437). Thus, EBP1 and EBP2 act in vivo to affect the function of E2F1 by altering its activity, in this case by repression. At present, it is not known how this repression takes place, but a number of possibilities arise. The effect could occur, for example, at the level of transcriptional activation, via an effect on the RNA polymerase II machinery (i.e., by affecting TBP/TAF, TFIIB, or TFIIH function). Also, given the fact that the EBP cDNAs are only partial length, the precise subcellular localization of their cognate proteins is not known at this time. It is possible that EBP1 and EBP2 could regulate E2F1 function in the perinuclear region or in the cytoplasm. However, given the effect of EBP1 and EBP2 on E2F1 transactivation of DNA pol α, they are likely to play a very important role in regulating other target gene expression by E2F1. In turn, regulation of target gene expression would potentially affect transit of a cell through the G1 and S phases of the cell cycle. Future experiments will be needed to better understand the role EBP1 and EBP2 play in the control of cell proliferation.

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