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ARTICLES

Regulation of the Pur-Alpha Promoter by E2F-1

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Abstract Purα is a ubiquitously expressed multifunctional nucleic acid-binding protein that is involved in many cellular processes including transcriptional regulation, the cell cycle, oncogenic transformation, and post-natal brain development. Previously, Purα protein was found to bind to E2F-1, inhibit E2F-1 transcriptional activity, and reverse the effects of ectopic E2F-1 expression on cell growth. Also Purα binds to a GC/GA-rich sequence within its own promoter and inhibits gene expression, that is, Purα is autoregulated. We now report that the Purα promoter (pPurα) is induced by E2F-1 and that this activity maps to a consensus E2F-1 binding motif that is juxtaposed to the Purα binding site. Deletion mutants of the E2F-1 protein showed that the region between amino acid residues 88–241 is important for this activity. E2F-1-associated activation of the pPurα was inhibited by co-expression of Purα, pRb, and an RNA species with specific binding to E2F-1. Chromatin immunoprecipitation (ChIP) assay using primers that flanked the juxtaposed Purα and E2F-1 binding sites verified the presence of Purα and E2F-1 on the pPurα in vivo. In a Tet-inducible cell line, Purα delayed cell cycle progression. Thus, E2F-1 and Purα interplay appears to be involved in the regulation of Purα expression and the cell cycle. J. Cell. Biochem. 99: 1052–1063, 2006.

Key words: Purα; E2F-1; promoter analysis; transcriptional regulation; cell cycle

Purα is a ubiquitously expressed multifunctional nucleic acid-binding protein that is involved in the initiation of DNA replication, control of transcription, and mRNA translation. Functional studies have implicated Purα as a major player in the regulation of the cell cycle and oncogenic transformation and it also has an essential role in post-natal brain development [Gallia et al., 2000; Johnson, 2003; Khalili et al., 2003]. Mouse Purα was originally purified from brain extracts as a protein that bound to the mouse myelin basic protein gene promoter [Haas et al., 1993, 1995] and human Purα was characterized by its ability to bind the c-Myc promoter [Bergemann and Johnson, 1992; Bergemann et al., 1992]. The sequence of mouse Purα [Ma et al., 1994] is almost identical to human Purα [Bergemann et al., 1992] with only 2 out of 322 amino acid residues differing. Purα has a distinctive modular structure with a central DNA-binding domain, and other notable structural features including an N-terminal glycine-rich domain and C-terminal glutamine-rich and glutamate-rich domains. The DNA-binding domain of Purα is strongly conserved throughout evolution. Purα is a member of the Pur family of proteins which also includes Purβ [Bergemann et al., 1992] and Purγ [Liu and Johnson, 2002], and it is expressed in virtually every metazoan tissue [Johnson, 2003]. Interaction of Purα with its recognition sequence, which is composed of repeats of (GGN), results in the formation of multimeric complexes and is modulated by the binding and interaction of other transcription factors [Gallia et al., 2000; Johnson, 2003].

Several lines of evidence suggest that Purα functions in the regulation of the cell cycle and oncogenic transformation. Purα binds to several cellular regulatory proteins including the retinoblastoma protein, pRb [Johnson et al., 1995], E2F-1 [Darbinian et al., 1999, 2004], Sp1 [Tretiakova et al., 1999], and YB-1 [Safak et al., 1999]. Some viral regulatory proteins target Purα and these include the Tat transactivator protein of the human immunodeficiency virus-1 [Gallia et al., 1999] and the large T-antigen of

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the human neurotropic polyomavirus JC [Gallia et al., 1998]. When microinjected into NIH-3T3 cells, Purα causes cell cycle arrest at either the G1/S or G2/M checkpoints [Stacey et al., 1999] and when expressed in Ras-transformed NIH-3T3 cells, Purα inhibits their ability to grow in soft agar [Barr and Johnson, 2001]. Ectopic overexpression of Purα suppresses the growth of several transformed and tumor cells including glioblastomas [Darbinian et al., 2001]. Deletions of Purα have been reported in myelodysplastic syndrome, a condition that can progress to acute myelogenous leukemia consistent with a role for Purα as a tumor suppressor [Lezon-Geyda et al., 2001]. Thus, Purα is an important transcription factor and regulator of the cell cycle that has a role in regulating cell proliferation. We have been interested in the interaction of Purα with E2F-1.

E2F-1 is a member of the E2F family of transcription factors implicated in the activation of genes required for the progression of cells into S-phase of the cell cycle. E2F-1 DNA binding sites are found in the promoters of several cellular genes implicated in S-phase entry including c-Myc, Cdc2, dihydrofolate reductase (DHFR), and DNA polymerase-α [Bracken et al., 2004]. The transcriptional activity of E2F-1 is negatively regulated by binding and sequestration within complexes with pRB. E2F-1 binds preferentially to hypophosphorylated pRB, which is believed to inhibit transit through the cell cycle. Hyperphosphorylation of pRB by cyclin-dependent kinases occurs as cells progress through G1 and this leads to pRb dissociation from E2F-1 and a concomitant increase in E2F-1 transcriptional activity [Frolov and Dyson, 2004]. Through this pathway, E2F-1 is a key positive mediator of cell cycle progression. However, E2F-1 also induces the expression of the protein $p14^{ARF}$, which interacts with MDM2/p53 causing p53 to be stabilized. This inhibits the cell cycle and promotes apoptosis [Bates et al., 1998; Vogelstein et al., 2000]. Thus, ectopic E2F-1 expression is growth-inhibitory [Melillo et al., 1994] especially when co-expressed with p53 [Wu and Levine, 1994] suggesting a role for E2F-1 in activating the p53 checkpoint [White and Khalili, 2004]. Activation of apoptosis appears to be unique to E2F-1 and reflects a specificity of transcriptional activation potential that is not found in the other E2F family members, for example, E2F-2 [Hallstrom and Nevins, 2003]. Thus, E2F-1 wields both positive and negative effects on cell proliferation.

E2F DNA-binding specificity to upstream non-transcribed regions of E2F-responsive genes has been characterized and is dependent upon the subunit composition of the heterodimeric transcription factor composed of E2F family members and DP-1 and DP-2 as well as Rb/E2F-1/DP-1 trimeric complexes [Tao et al., 1997]. In addition, E2F-1 has also been demonstrated to bind to single-stranded RNA [Ishizaki et al., 1996] through a GGAGAGAG consensus sequence. Binding to this motif has been demonstrated in RNA gel shift assays and ectopic expression of RNA containing this motif inhibits E2F activity [Darbinian et al., 2004]. This motif is found near the 5′ end of the Purα primary transcript in close juxtaposition to the site where Purα has been shown to bind, which is located close to the transcription start site and mediates Purα autoregulation of its own promoter [Muralidharan et al., 2001].

From the studies described above, it is apparent that both E2F-1 and Purα are transcription factors with important roles in the regulation of cell proliferation. Our interest has focused on the interaction of these two proteins. In previous studies, we demonstrated that Purα binds directly to E2F-1 [Darbinian et al., 1999]. Purα did not bind to the E2F-binding sites in double-stranded DNA but it caused downregulation of transcription from a DHFR-luciferase reporter construct (containing E2F-binding sites) by virtue of the ability of Purα to bind and sequester E2F-1 [Darbinian et al., 1999]. The N-terminal domain of Purα (residues 1–72) was found to be important for in vitro binding to E2F-1, inhibition of E2F-1 DHFR-luciferase transcription, and reversal of E2F-1 inhibition of cell proliferation in a series of experiments involving Purα protein deletion mutants [Darbinian et al., 2004]. Thus, E2F-1/Purα interaction has a role in the control of E2F-1 function and cell proliferation.

Here, we report the characterization of the nature and the effects of the interaction between Purα and E2F-1 on transcription from the Purα promoter (pPurα). This study was prompted by two observations. Firstly, Purα binds to a GC/GA-rich sequence that is located close to the transcription start site within its own promoter and inhibits gene expression, that is, Purα is autoregulated [Muralidharan et al., 2001]. Secondly, ectopic expression of

E2F-1 in glial cells increased the level of Purα detected by Western blot [Darbinian et al., 1999]. Therefore, we explored the hypothesis that interplay between Purα and E2F-1 might regulate expression of the pPurα.

MATERIALS AND METHODS

Plasmids and Synthetic Oligonucleotides

pCDNA6-B-Purα expresses Purα with a Myc-His tag from the CMV promoter. It was constructed by subcloning a *Xho*I–*Bam*HI fragment containing the Purα coding region from EBV-Purα [Gallia et al., 1998] into *Xho*I/*Bam*HI site of pCDNA-6 Myc/His-B (Invitrogen, Carlsbad, CA). pCMV-Purα, which contains untagged full-length Purα in the pCDNA3 eukaryotic expression vector, and pCMV-E2F-1 have been described previously [Darbinian et al., 1999]. pCMV-E2F-2 was a kind gift from Dr. Antonio Giordano (Sbarro Institute for Cancer Research and Molecular Medicine, Temple University). pCMV-E2F-1 deletion mutant variants were kindly provided by Dr. Dave Hall. The plasmids pCDNA-E-RNA and pCDNA-P-RNA have been described previously [Darbinian et al., 2004]. Luciferase reporter plasmids containing the mouse pPurα and promoter deletion mutants have been described previously [Muralidharan et al., 2001] except for pPurα (δ5′UTR). This was made by PCR amplifying the portion of the promoter without the 5′UTR from pPurα (1090 bp), cloning into pCR-TII (Invitrogen) and subcloning the 1.1-Kb δ5′UTR promoter fragment into the *Sma*I site of pGL3 (Promega, Madison, WI). pDHFR-Luc expresses the luciferase reporter gene under the control of the DHFR promoter (pDHFR) and was kindly provided by Dr. Peggy Farnham (McArdle Laboratories, University of Wisconsin, Madison, WI). pTRE-GFP was made by cloning the GFP from pLEGFP-C1 (Clontech, Palo Alto, CA) in front of the Tet response element of pTRE (Clontech). pTRE-GFP-Purα was made by cloning Purα from pCDNA6-Purα into pLEGFP-C1 and then subcloning the GFP-Purα fusion gene into pTRE. The sequence of all plasmids was verified by DNA sequencing using an ABI automatic sequencer.

Antibodies

Anti-E2F-1 (C-20) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Myc antibody was obtained from Invitrogen. Mouse monoclonal anti-Grb2 antibody was from BD Transduction laboratories (Lexington, KY). Anti-Purα was a mouse monoclonal antibody (clone 10B12) kindly provided by Dr. Ed Johnson (Mount Sinai, New York, NY).

Cell Culture and Transfection

U-87 MG human glioblastoma cells and mouse NIH-3T3 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) and antibiotics (100 units/ml penicillin and 10 μg/ml streptomycin) at 37°C in a humidified atmosphere containing 7% CO_2.

Transfections were carried out by the calcium phosphate technique as described [Graham and van der Eb, 1973], using U-87 MG cells. In brief, 2×10^5 cells were plated on a 60-mm plate and grown overnight. Transfection was carried out with 10 μg of DHFR-luciferase reporter DNA or 5 μg of full-length or mutant pPurα-luciferase reporter plasmids and co-transfected with 2.5, 5, or 10 μg of the following expression plasmids as indicated in the figures: pCMV-Purα, pCMV-E2F-1 and its deletion mutants, pCMV-pRB, pCDNA-P-RNA, and pCDNA-E-RNA. Vector plasmid was added to each transfection to equalize the total amount of DNA in each transfection mixture. The precipitates were removed after 3 h and a glycerol shock was applied. Thirty-six hours post-transfection, the cells were harvested and a crude protein extract was prepared by lysis in reporter lysis buffer according to the protocol of the manufacturer (Promega). Extracts were quantitated by the Bradford assay (Bio-Rad Laboratories, Richmond, CA), and equal amounts of proteins (4 μg) were assayed for luciferase activity. Each transfection was repeated a minimum of two or three times.

Chromatin Immunoprecipitation (ChIP) Assay

U-87 MG cells were plated in 100-mm tissue culture dish (Falcon) and transfected with pCDNA6-B-Purα or pCMV-E2F-1. Some cultures were treated with mitoxantrone at a final concentration of 50 nM. After 36 h, cells were washed with cold phosphate-buffered saline (PBS) and then cross-linked with 1% formaldehyde at 37°C for 10 min. Cells were lysed solubilized in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Nonidet

P-40, and 1% protease inhibitors cocktail (Sigma)), sonicated and supernatants were then recovered by centrifugation of lysate at 14,000 rpm for 5 min at 4°C to remove cell debris. The supernatant was assayed for protein content by Bradford and whole cell lysates were used for immunoprecipitations. Immunoprecipitation of complexes was carried out by incubating approximately 250 μg of precleared cell lysates with either 5 μl of anti-E2F-1 or anti-Myc for 16 h at 4°C. Then, immune complexes were precipitated with 30 μl of protein A-Sepharose for 2 h at 4°C and washed with PBS. Cross-linking was reversed by heating for 2 h at 65°C in 200 mM NaCl. DNA was extracted from the immune complexes with phenol–chloroform, ethanol precipitated, air dried, and dissolved in 50 μl of water. Five microliters of DNA sample was subjected to PCR amplification utilizing primers corresponding to the promoter region of Purα on human chromosome 5 (5′-tacagtagggcgccctgctactgtac-3′ and 5′-gatgctgcgctccgctgccg-3′). One of three of the total cell lysate supernatant was used for the positive control PCR (input DNA), after phenol–chloroform extraction and sodium acetate–ethanol precipitation of DNA. For PCR, 32 cycles were performed with an annealing temperature of 62°C.

Production of U-87 MG-Derived Stable Cell Lines Expressing Inducible GFP-Purα and GFP

Firstly, a stable cell line was made with the pTet-On plasmid (Clontech). U-87 MG cells were transfected with pTet-On and then cells were selected by transferring to medium containing G418 (600 μM) 24 h after transfection. After 3 weeks, 10–15 G418-resistant clones were isolated by dilution cloning and screened for inducibility by transient transfection with pTRE-luciferase for clones with low background and high doxycycline-dependent induction after cells were induced with 2 μg/ml doxycycline for 16 h, and analyzed for luciferase assay. In the second round of stable transfection, cells of the selected Tet-On clone were co-transfected with pTRE-GFP-Purα or pTRE-GFP and pCDNA6-Myc/His-B for selection of blasticidin-resistant clones. Cells were maintained for 3 weeks in DMEM containing fetal bovine serum, G418 (0.6 mM), and blasticidin (5 μg/ml) for double selection, after which, 10 clones were plated, cultured in the absence or presence of 2 μg/ml doxycycline for 16 h, and each clonal cell line was studied for visualization of GFP fluorescence with an inverse fluorescence microscope equipped with a FITC filter set, using 20× objectives. Positive clones were also tested for inducibility of GFP or GFP-Purα expression in Western blot analysis, using polyclonal antibody to GFP (Clontech).

Cell Cycle Analysis

Cells expressing inducible GFP-Purα and GFP were incubated in serum-free medium for 48 h, and then changed to medium containing 10% FBS and 2 μg/ml doxycycline. At different time points ranging from 4 to 28 h, cells were fixed in 88% ethanol at −20°C, pelleted and stained with propidium iodide (PI)-RNase A solution for 30 min at 37°C. FACS analysis to determine cell cycle distribution was performed with a FACSORT Flow cytometer (Becton Dickson) using Cell Fit Software, vs. 2.01.2 (Becton Dickson). FACS analysis data were derived from counting at least 20,000 events in each sample.

RESULTS

Mapping of the E2F-1 Response Element of the Mouse Purα Promoter to the 5′UTR

Previous studies in this laboratory identified a GA/GC-rich sequence near the transcription start site of the mouse Purα gene that is responsible for autoregulation of the pPurα activity [Muralidharan et al., 2001]. An E2F-1 consensus RNA binding site motif [Ishizaki et al., 1996] is located at the 5′ end of the 5′UTR UTR in close juxtaposition to this site in the mouse promoter (Fig. 1). Note that similarly juxtapositioned Purα and E2F-1 sites are also found in the human pPurα. We assayed a series of luciferase reporter constructs containing various regions of the Purα for transcriptional activity in the presence and absence of exogenously expressed E2F-1 in U-87 MG cells (Fig. 1). The construct containing the full-length 6,000-bp pPurα was induced 3.5-fold by co-expression of E2F-1. Deletion of the promoter from its 5′ end to give constructs with 2,300 bps and 1,090 bps resulted in a reduction in E2F-1 inducibility. However, the construct containing the 210 bp of the 3′ end of the pPurα was 2.6-fold inducible by E2F-1 but E2F-1 inducibility was abolished by deletion of the 5′UTR. Note that the number for each construct indicates the distance in base pairs from the 5′ end of the

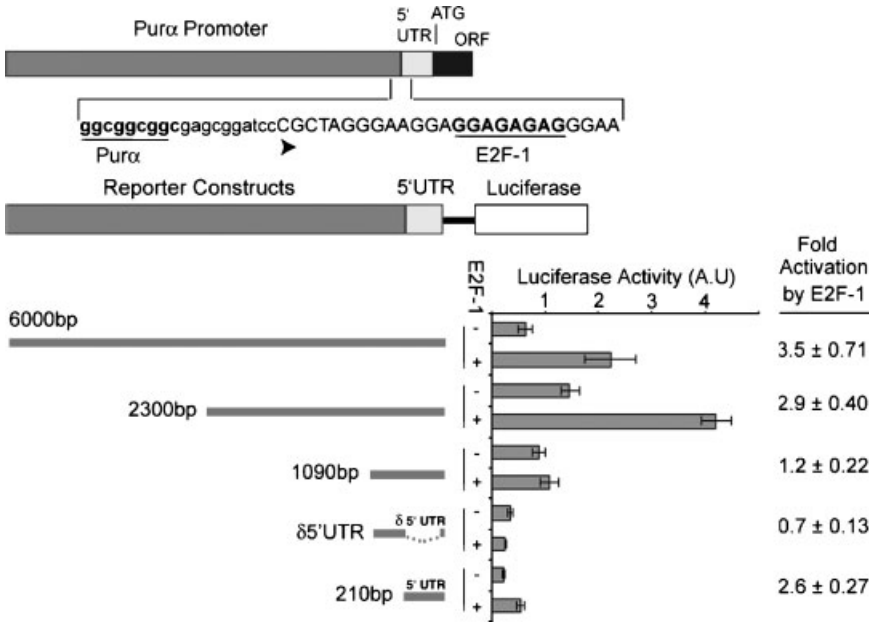

Fig. 1. Stimulation of the pPurα by E2F-1 and localization of E2F-1 binding site. The pPurα is shown with the region containing the juxtaposed Purα and E2F-1 binding sites indicated by the vertical lines. The sequence between the vertical black lines, which spans the transcription start site (indicated by an arrowhead), is presented with transcribed nucleotides in capitals and the Purα and E2F-1 consensus binding sites are indicated. Below this is shown the structure of the plasmid constructs containing various portions of the pPurα and their relative stimulation of luciferase activity by E2F-1. This was performed as follows. U-87 MG cells were transfected with five different constructs containing various regions of the pPurα in the presence and absence of plasmid expressing E2F-1 as described in Materials and Methods. All constructs were created by digesting the pPurα with restriction endonucleases and retain the part of the pPurα indicated by a line. Further details of these constructs are given in Materials and Methods and in reference [Muralidharan et al., 2001]. The histogram shows mean luciferase activity and the error bars show the standard deviation. The lower right-hand side lists the fold stimulation by E2F-1 (± standard deviation) for each of the Purα-luciferase constructs. This experiment was performed three times.

promoter construct to the Purα ATG translation initiation codon and includes the 135-bp 5′UTR except for the construct pPurα (δ5′UTR) in which the 5′UTR is deleted. Thus, the 5′UTR, which contains the consensus binding site motif for E2F-1, confers E2F-1 inducibility in this experiment. Subsequent experiments with the pPurα utilized the 2,300-bp construct.

We also analyzed the effect of another E2F family member on the pPurα constructs, E2F-2 which, unlike E2F-1, is not pro-apoptotic. We obtained essentially the same results, that is, E2F-2 inducibility was seen in all the constructs except pPur (δ5′UTR) in which the 5′UTR is deleted (Table I).

Deletion Analysis of the E2F-1 Protein

We used deletion mutants of E2F-1 to delineate the domain(s) within the E2F-1 transcription factor that is involved in induction of the pPurα (Fig. 2A) and to compare this with results obtained for the pDHFR (Fig. 2B). When co-transfected into U-87 MG cells, full-length E2F-1 stimulated the Purα and pDHFRs about four–fivefold (Fig. 2). The construct with the N-terminal 88 amino-acid residues deleted retains its ability to stimulate both promoters. The construct (88–241) has both the N-terminal 88 amino-acid residues deleted and C-terminal 196 amino-acid residues deleted, and retains the central DNA binding domain. Interestingly, this mutant strongly stimulated the pPurα

TABLE I. Stimulation of the pPurα by E2F-2

Construct	Luciferase activity −E2F-2	Luciferase activity +E2F-2	Fold activation by E2F-2
pPur (6,000)	1.00 ± 0.305	3.25 ± 0.212	3.84 ± 0.959
pPur (2,300)	1.01 ± 0.017	1.91 ± 0.175	1.88 ± 0.14
pPur (1,090)	0.64 ± 0.032	2.14 ± 0.729	3.23 ± 0.975
pPur (δ5′UTR)	0.26 ± 0.055	0.23 ± 0.038	0.90 ± 0.048
pPur (210)	4.12 ± 0.693	8.73 ± 1.05	2.15 ± 0.108

U-87 MG cells were transfected with five different constructs containing various regions of the pPurα in the presence and absence of plasmid expressing E2F-2 and luciferase activity determined as described in Materials and Methods. This experiment was performed twice.

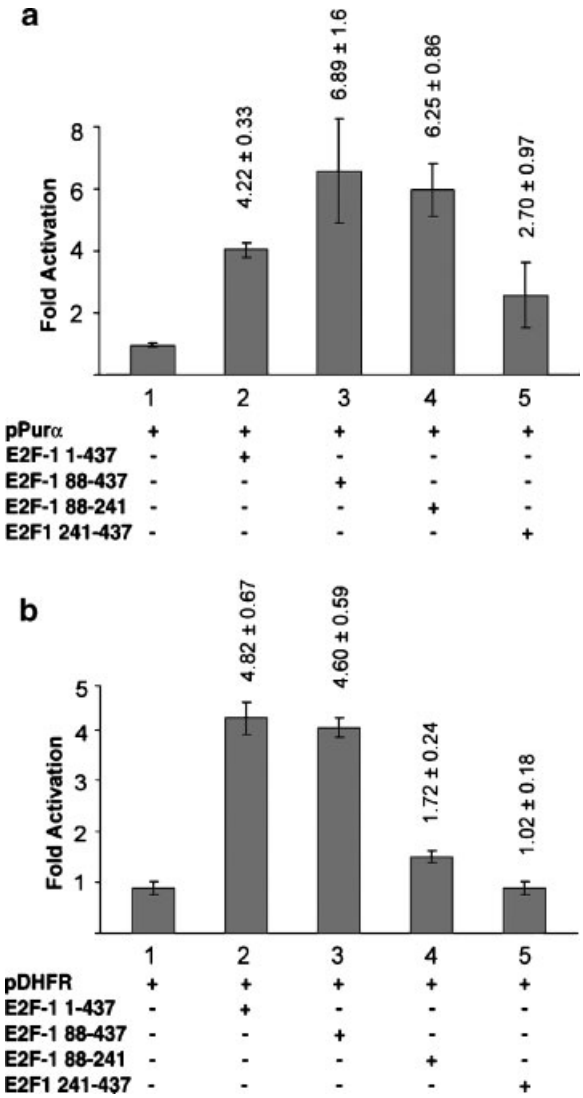

Fig. 2. Activation of the Purα and pDHFR by E2F-1 and E2F-1 deletion mutants. Panel **a**. *Activation of the pPurα.* U-87 MG cells were transfected with luciferase reporter plasmids containing the mouse pPurα 2300 in the presence or absence of plasmid expressing E2F-1 or E2F-1 deletion mutants as indicated. Panel **b**. *Activation of the DHFR promoter.* U-87 MG cells were transfected with luciferase reporter plasmids containing the mouse pDHFR in the presence or absence of plasmid expressing E2F-1 or E2F-1 deletion mutants as indicated. This experiment was performed twice.

(6.25-fold) but was significantly weaker ($P = 0.004$) in stimulating the pDHFR (1.72-fold).

When the effect of the E2F-1 deletion mutants on the activity of the pPurα was measured in the presence of co-expressed Purα, it was found that E2F-1 stimulation was abrogated for all of the mutants (Fig. 3, compare lanes 6–11 with lanes 1–5). Expression of reporter activity in all of the E2F-1 lanes, which co-expressed Purα (Fig. 3, lanes 6–11) was lower than basal pPurα expression (Fig. 3, lane 1).

Role of Rb in Purα Promoter Regulation

As well as binding to Purα [Darbinian et al., 1999, 2004], E2F-1 binds to the retinoblastoma protein, pRb [Frolov and Dyson, 2004]. Purα can also bind to pRb [Johnson et al., 1995]. Thus, it was of interest to study the effect of ectopic pRb expression on the activity of the pPurα. When Rb was co-expressed in U-87 MG cells with the pPurα, the activity of the promoter was decreased by 72% (Fig. 4A, compare lanes 1 and 7), which is similar to the inhibition of expression (70%) observed when Purα was co-expressed in these cells (Fig. 4A, compare lanes 1 and 3). However, the inhibitory effect of Rb was not observed if E2F-1 was also co-expressed in the same cells (Fig. 4B, compare lanes 4 and 5 with lanes 8 and 9). In the same experiment, it was found that increasing amounts of E2F-1 reversed the inhibitory effect of pRb expression (Fig. 4B, compare lanes 1, 10, and 11).

Previously, we reported the construction of the plasmids pCDNA-E-RNA and pCDNA-P-RNA that express oligoribonucleotides that specifically bind to E2F-1 and Purα, respectively [Darbinian et al., 2004]. Both of these RNA species disrupt the interaction of Purα and E2F-1 and reverse the growth inhibition of cells that express ectopic Purα [Darbinian et al., 2004]. When these RNA species were expressed alone in cells, there was no effect on the activity of the pPurα (Fig. 4C, compare lanes 1, 4, and 5). However, E-RNA was able to inhibit the stimulatory effect of E2F-1 on the pPurα (Fig. 4C, compare lanes 3 and 6). Similarly, P-RNA was able to relieve the inhibitory effect of Purα on the pPurα (Fig. 4C, compare lanes 2 and 9).

Purα and E2F-1 Associate With the Purα Promoter in Vivo

To investigate the association of Purα and E2F-1 with the pPurα in living U-87 MG cells, we employed ChIP assays (Fig. 5). Cells were transfected with plasmids expressing E2F-1 and/or Myc-tagged Purα and/or treated with mitoxantrone, a drug that binds to GC residues in DNA and interferes with E2F-1-DNA complex formation [Chiang et al., 1998]. Cell cultures were cross-linked, immunoprecipitations were performed with antibodies for Purα (anti-Myc) and E2F-1 and PCR executed using primers flanking the region from the

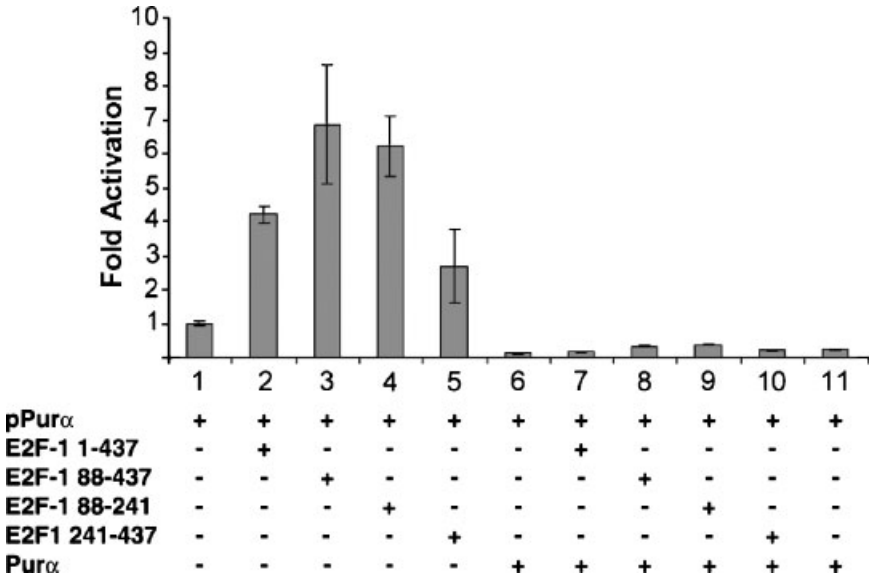

Fig. 3. Effect of co-expressed Purα on the activation of the pPurα by E2F-1 and E2F-1 deletion mutants. U-87 MG cells were transfected with luciferase reporter plasmids containing the mouse pPurα 2300 in the presence or absence of plasmid expressing E2F-1 or E2F-1 deletion mutants and/or plasmid expressing Purα as indicated.

pPurα containing the juxtapositioned Purα and E2F-1 sites. The positive control of input cell extract was positive for all extracts (Fig. 5, top panel) while the negative control, immunoprecipitation with normal mouse serum (Fig. 5, second panel), showed no bands in any of the lanes. Cells transfected with plasmid expressing Purα were positive with Purα immunoprecipitation and this was only slightly inhibited by mitoxantrone (Fig. 5, third panel, compare lanes 3 and 4 with lanes 7 and 8). All cell cultures that were not treated with mitoxantrone were positive for E2F-1 immunoprecipitation and the signal was increased in cells that were transfected with the E2F-1-expressing plasmid (Fig. 5, bottom panel, compare lanes 1 and3 with lanes 2 and 4). Binding of E2F-1 to the Purα was much reduced, but not completely eliminated in cultures that were treated with mitoxantrone (Fig. 5, bottom panel, lanes 5–8).

Induction of Purα Expression Retards the Cell Cycle

In order to investigate the result of Purα expression we constructed a stable cell line, GFP-Purα from U-87 MG cells in which expression of GFP-Purα was inducible by doxycycline (Tet-On system). As a control, we used a parallel cell line, which was inducible for expression of GFP alone. Cells were synchronized in G0 with serum-free medium and then stimulated by changing to medium containing serum and doxycycline. As shown in Figure 6, cells expressing GFP alone progressed normally through the cell cycle and about 48% had reached S-phase after 16 h with the G0/G1 population falling from 64% to about 37%. In contrast, cells that expressed GFP-Purα were largely arrested in G0/G1. Only 16% of the GFP-Purα cells were in S-phase after 16 h and the G0/G1 population falling only slightly (from about 79% to about 73%). Thus, induction of Purα expression has a negative effect of cell cycle progression through G1-phase to S-phase in human glioblastoma cells.

Purα Expression Decreases at the End of G1

NIH-3T3 cells were synchronized in G0 by serum starvation for 2 days and then stimulated to enter the cell cycle by the addition of complete medium containing serum. Total cell proteins were harvested at various time points and analyzed by Western blot for Purα expression. The level of Purα protein remained constant at 4 and 16 h, but dropped at 21 h (Fig. 7A). Purα returned to a higher level at 29 h and later. E2F-1 showed the opposite pattern of expression and was increased at 21 h. The Western blot in Figure 7A was quantitated by densitometry of the intensity of the Purα lanes relative to the

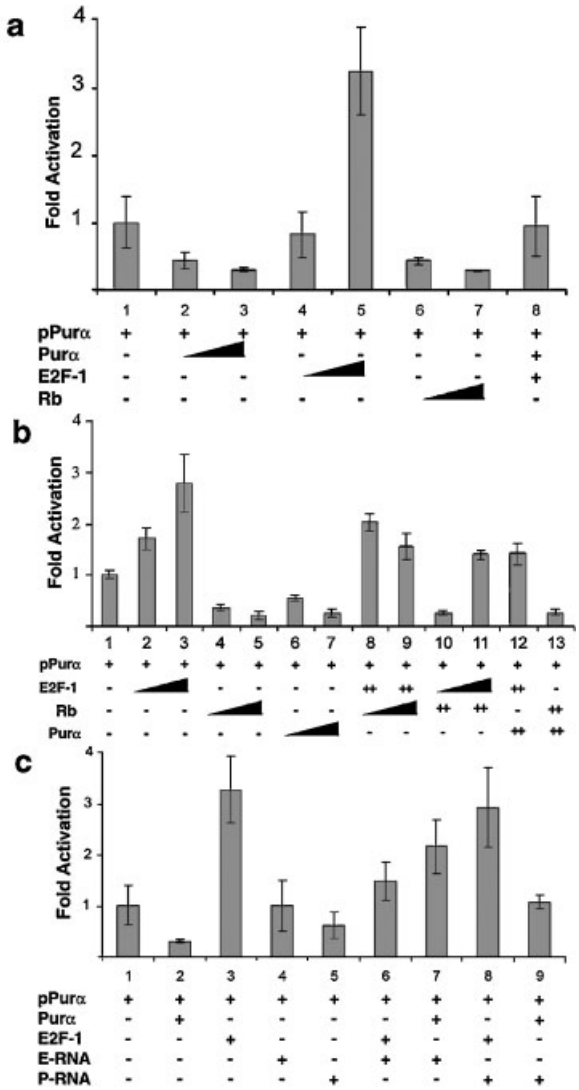

Fig. 4. Effect of co-expressed Rb, E-RNA, and P-RNA on the activation of the pPurα by E2F-1. U-87 MG cells were transfected with luciferase reporter plasmids containing the mouse pPurα in the presence or absence of plasmid expressing E2F-1 and/or plasmid expressing Purα, pRb (**a**, **b**) E-RNA, and P-RNA (**c**) as indicated. Plasmids that were used at low (2.5 μg) and high (10 μg) DNA concentrations are indicated by the ramp symbol (i.e., 2.5 μg 10 μg). In Panel **b**, ''++'' indicates 10 μg of plasmid DNA.

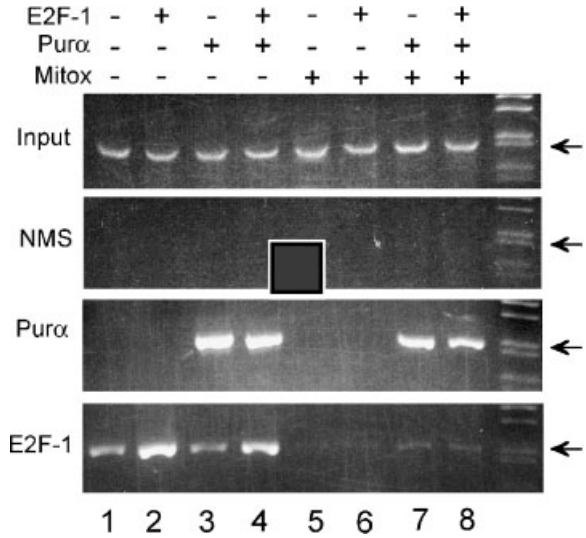

Fig. 5. ChIP assay for Purα and E2F-1 at the pPurα. U-87 MG cells were transfected with pCMV-E2F-1 and/or pCDNA6-B-Purα and treated with and without mitoxantrone as indicated in the header. The top panel shows the input DNA (positive control) and the second panel shows immunoprecipitation with normal mouse serum (negative control). The last two panels show immunoprecipitation of Purα and E2F-1. The arrows indicate the position of the PCR product (221 base pairs).

Grb2 loading control lanes (Fig. 7B). Cell cycle analysis showed that the 21-h time point (lane 4) corresponded to the maximum of G2/M cells and the minimum of G1/G0 cells (Fig. 7C).

DISCUSSION

E2F-1 is a critical regulator of the cell cycle that activates transcription of many genes encoding proteins that are necessary for cell cycle progression into S-phase but can also promote apoptosis in some circumstances [Bates et al., 1998; Vogelstein et al., 2000; Hallstrom and Nevins, 2003]. Regulation of E2F-1 is complex and involves interactions with at least two other proteins, Purα and pRb, which themselves can form a complex. In addition, E2F-1 can positively regulate transcription from its own promoter [Neuman et al., 1994] and Purα can negatively regulate transcription from its own promoter [Muralidharan et al., 2001]. Here, we report that E2F-1 activates transcription from the pPurα. This web of interactions is depicted in Figure 8.

Interestingly, the E2F-1 site that activates pPurα transcription and the juxtapositioned autoregulatory Purα-binding site span the start site of transcription as shown in Figure 1. The degree of E2F-1 induction of the minimal construct containing these sites, pPurα (210 bp), is induced to almost the same extent (2.6-fold) as the full-length construct, pPurα (6,000 bp), (3.5-fold). The absolute level of expression of the full-length construct is higher than the minimal construct and this is likely due to the stimulatory effect of other transcription factors, which bind to the deleted region.

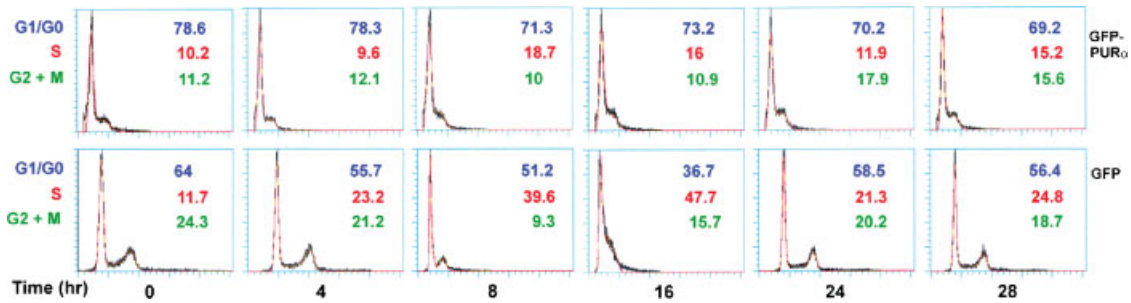

Fig. 6. Cell cycle analysis of cells expressing inducible Purα. A Tet-on cell line that expresses GFP-Purα in response to doxycycline and a control cell line expressing inducible GFP were produced as described in Materials and Methods. After serum starvation, cells were transferred to complete medium containing doxycycline and harvested at the time points indicated.

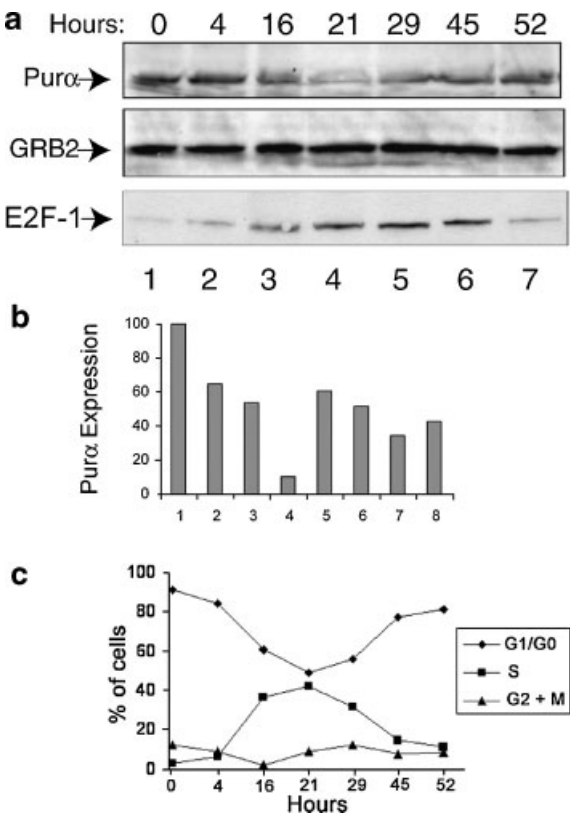

Fig. 7. Purα expression during the cell cycle. Panel **a**. *Western Blot.* NIH-3T3 cells were serum starved and then returned to complete medium. At the time points indicated, cells were harvested for total cell protein. Western blot analysis was performed for Purα expression. Grb2 was used as a loading control. The expression of E2F-1 was also measured by Western blot. Panel **b**. *Quantitation of Purα Western blot.* The data in Panel A were quantitated using Quantity One software (Bio-Rad) normalizing first to the Grb2 level for each lane and then relative to **lane 1** (time zero) as 100%. Panel **c**. *FACS.* Cell cycle analysis of the cells after return to complete medium.

It is important to note that the mechanism of induction of the pPurα may be different from that of other promoters, for example, DHFR and cyclin E, where E2F-1 binds to an upstream non-transcribed double-stranded DNA sequence with a TTTCGCGC motif [Tao et al., 1996]. Rather the E2F-1 site in the Purα is immediately downstream of the Purα transcription start site and contains a high affinity RNA-binding GGAGAGAG motif [Ishizaki et al., 1996]

Whereas a region spanning the central and C-terminal domains of E2F-1 (88–437) is required

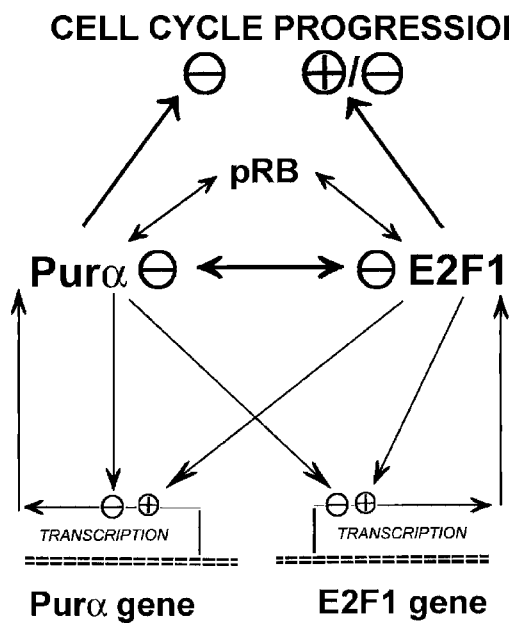

Fig. 8. Schematic representation of protein interactions. The interactions that are known to occur between transcription factors are shown. ''−'' denotes an inhibitory interaction, ''+'' denotes a stimulatory interaction. ''+/−'' signifies that E2F-1 can promote cell cycle progression but can also promote apoptosis.

to activate transcription from the pDHFR, only the central region (88–241) containing the DNA-binding domain is required to activate transcription of the pPurα (Fig. 2). This may be due to differences in the mechanism of E2F-1 transcriptional activation. Alternatively, it may be due to DNA sequence-specific differences between the promoters affecting E2F-1 binding or to the interaction of E2F-1 with transcription factors that bind to adjacent sites that differ between the two promoters. Co-expression of Purα, which binds close to E2F-1 in the pPurα, abrogates transcriptional stimulation by full-length E2F-1 and all of its deletion mutants (Fig. 3).

Clearly the stimulation of the pPurα by E2F-1 is downregulated by pRb (Fig. 4). pRb can bind to both E2F-1 and to Purα. The negative regulation of E2F-1 activity that occurs when it interacts with pRb may be responsible for the negative effect of pRb expression on E2F-1's effect on the pPurα. Also of interest is the possible role of other E2F-1 family members at the pPurα. The E2F family of transcription factors currently has eight members (E2Fs 1–8), which can have positive and negative effects on transcription. Studies on mutant mouse models lacking E2Fs 1–6 have revealed functional redundancies, for example, between E2Fs 1–3 acting upon genes involved in cell proliferation, as well as unique roles, such as the specific role for E2F-1 in apoptosis [reviewed in Attwooll et al., 2004]. Interestingly, E2F-2, which is an E2F family member that does not possess pro-apoptotic activity, also stimulates Purα transcription except in the construct where the 5′UTR is deleted (Table I). It would thus be of interest to determine if other E2F family members can act at the pPurα.

Interestingly, the E-RNA and P-RNA species, which bind to E2F-1 and Purα, reverse the effects of their respective binding proteins on the pPurα (Fig. 4C). While E-RNA is an RNA derived from a library [Ishizaki et al., 1996], P-RNA represents a sequence that is known to exist in U-87 MG cells based on RT-PCR and sequence analysis of RNA that co-immunoprecipitated with Purα from U-87 MG nuclear extracts [Tretiakova et al., 1998; Gallia et al., 1999]. Thus, it is likely that regulation of the activity of Purα by P-RNA is a physiologically relevant event.

Both E2F-1 and Purα bind to the pPurα in vivo as shown by ChIP assay (Fig. 5). These interactions were inhibited by treatment of the cells with mitoxantrone, a drug that binds to GC residues in DNA and interferes with E2F-1-DNA complex formation [Chiang et al., 1998]. While only slight inhibition was observed for Purα, binding of E2F-1 was almost completely inhibited by mitoxantrone.

Expression of Purα in a tetracycline-inducible cell line inhibited progression of the cell cycle (Fig. 6). This is in agreement with other research demonstrating a negative role for Purα on cell growth and that Purα can act as a tumor suppressor [Stacey et al., 1999; Barr and Johnson, 2001; Darbinian et al., 2001; Lezon-Geyda et al., 2001]. Thus, E2F-1 stimulation of Purα expression may represent a feedback loop serving to moderate the activity of E2F-1 during progression of the G1-phase of the cell cycle since Purα directly binds and inhibits E2F-1 [Darbinian et al., 2004]. In agreement with this idea, the expression level of Purα measured at the protein level by Western blot during the cell cycle (Fig. 7) is high during G1 progression, drops in S and G2, and rises again after cells pass through M-phase. Interaction of E2F-1 with Purα is an important mechanism for controlling the activity of E2F-1 and hence, regulating cell cycle progression [Darbinian et al., 2004]. Since E2F-1 regulates the transcription of the Purα gene, this may provide another means of fine-tuning the regulation of E2F-1. Thus, E2F-1 and Purα form a complex regulatory network together with pRb as depicted in the schematic diagram shown in Figure 8. This network co-ordinates gene expression and cell cycle progression.

Finally, it should be noted that E2F family members have been implicated in cell cycle exit and differentiation as well as cell cycle progression [Attwooll et al., 2004]. Recently, we created transgenic mice with inactivation of the PURA gene that encodes Purα, and observed that Purα has an essential role in post-natal brain development [Khalili et al., 2003]. $PURA^{-/-}$ mice appear normal at birth, but at 2 weeks of age, they develop neurological problems and they die by 4 weeks. This is due to a lack of proliferation of precursor cells in the brain cortex, hippocampus, and cerebellum. This implicates Purα in the regulation of developmentally timed DNA replication in specific cell types in the brain. Thus, developmental regulation of the pPurα by E2F family members may be important in processes involved in neural cell differentiation.

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