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Interaction Between the Purα and E2F-1 Transcription Factors

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Abstract. *Background: Cell proliferation is regulated by E2F-1 which facilitates expression of genes involved in entry into S-phase. Release of E2F-1 from binding partners, e.g. pRb, is critical for G1/S progression. However ectopic E2F-1 overexpression activates p53 and inhibits growth. Previously, the multifunctional Purα protein was found to bind to E2F-1 and inhibit E2F-1 transcriptional activity. Materials and Methods: Purα deletion mutants were assayed for: in vitro binding to E2F-1, inhibition of E2F-1-induced promoter activation and effects on cell proliferation. Two RNA species with specific binding to E2F-1 and Purα were analyzed for their effects on E2F-1/Purα binding and cell growth. Results: The N-terminal 72 amino acids of Purα were involved in E2F-1 binding, inhibition of promoter activation by E2F-1 and reversal of E2F-mediated growth inhibition. The RNA species disrupted Purα/E2F-1 interaction and affected cell growth. Conclusion: E2F-1/Purα interaction has a role in the control of cell proliferation.*

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 the S-phase of the cell cycle. E2F DNA binding sites have been found in the promoters of several cellular genes implicated in S-phase entry, including c-Myc, Cdc2, dihydrofolate reductase (DHFR) and DNA polymerase-α (1). The transcriptional activity of E2F proteins is negatively regulated by their binding and sequestration within complexes with the retinoblastoma family of growth regulatory proteins. Within this family, the interaction of E2F-1 and pRB, the product of the RB-1 retinoblastoma susceptibility gene, has been examined in greatest detail. 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 (2-6). Through this pathway, E2F-1 is a key positive mediator of the effects of the pRb regulator on cell cycle progression.

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 (6,7). Thus ectopic E2F-1 expression was found to be growth-inhibitory in colony-forming assays (8) and this effect is magnified by coexpression of the wild-type p53 protein (9). It is likely that the activation of the p53 checkpoint by E2F-1 overexpression represents a defense mechanism against inappropriate loss of pRb activity, *e.g.*, after infection by certain DNA tumor viruses (10). 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 (11). Thus E2F-1 wields both positive and negative effects on cell proliferation.

Ishizaki *et al.* (12) reported the isolation of RNA species from a high-complexity RNA library that were selected on the basis of their ability to bind to the DNA-binding site of the E2F-1 protein. These RNAs inhibited binding of E2F-1 to the E2F DNA recognition sequence in gel shift assays and were growth inhibitory upon microinjection into human fibroblasts (12).

Purα is a ubiquitous nucleic acid-binding protein that was originally purified from mouse brain based on its ability to bind to a DNA sequence derived from the promoter of the mouse myelin basic protein gene (13,14). Human Purα was characterized by its ability to bind to a DNA sequence

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present upstream of the human c-Myc gene and its cDNA was cloned from HeLa cells and sequenced (15,16). The sequence of mouse Purα (17) is almost identical to human Purα (16) with only 2 out of 322 amino acid residues differing. The DNA-binding domain of Purα is strongly conserved throughout evolution.

Purα is a member of the Pur family of proteins along with Purβ (16) and Purγ, for which there exist two isoforms that arise from the usage of alternative polyadenylation sites (18), and it is expressed in virtually every metazoan tissue (19). Purα is a multifunctional protein that can bind to both DNA and RNA and functions in the initiation of DNA replication, control of transcription and mRNA translation (19,20). Purα associates with DNA sequences that are close to viral and cellular origins of replication. Since initiation of transcription and replication requires unwinding of duplex DNA, this is consistent with Purα being a single-stranded nucleic acid-binding protein that possesses DNA helix-destabilizing activity (21).

Several lines of evidence suggest that Purα is a major player in the regulation of the cell cycle and oncogenic transformation. Purα binds to several cellular regulatory proteins including the retinoblastoma protein (22), E2F-1 (23), Sp1 (24) and YB-1 (25). Some viral regulatory proteins target Purα and these include the Tat transactivator protein of the human immunodeficiency virus-1 (26) and the large T-antigen of the human neurotropic polyomavirus JC (27). The intracellular level of Purα varies during the cell cycle, declining at the onset of S-phase and peaking during mitosis (28). When microinjected into NIH-3T3 cells, Purα causes cell cycle arrest at either the G1/S or G2/M checkpoints (29) and when expressed in Ras-transformed NIH-3T3 cells, Purα inhibits their ability to grow in soft agar (30). Ectopic overexpression of Purα suppresses the growth of several transformed and tumor cells including glioblastomas (31). The growth-inhibitory effects of Purα are consistent with gene expression profiling in chronic myeloid leukemia patients where down-regulation of Purα expression was observed (32). Furthermore, 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 (33).

Thus both E2F-1 and Purα are transcription factors with key roles in the regulation of cell proliferation. In previous studies, we demonstrated that Purα binds directly to E2F-1 in nuclear extracts (23). Purα did not bind to the E2F-binding sites in double-stranded DNA but it caused down-regulation 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 (23). Here we report the further characterization of the nature and the effects of the interaction between Purα and E2F-1.

Materials and Methods

Plasmid constructs and synthetic oligonucleotides. The following plasmids have been described previously: GST-Purα and its deletion mutant variants (27), pCDNA-Purα which contains full-length Purα (26) and its deletion mutant variants in the pCDNA3 eukaryotic expression vector (Invitrogen, Carlsbad, CA, USA) expressed from the CMV promoter, GST-E2F-1 (34) and pCDNA-E2F-1 (35), pEBV-Purα which contains the coding region of the Purα gene and its deletion mutant variants cloned downstream of a T7/Histidine epitope tag (27). DHFR-Luc expresses the luciferase reporter gene under the control of the DHFR promoter and was kindly provided by Dr. Peggy Farnham (McArdle Laboratories, University of Wisconsin, Madison, WI, USA).

The following synthetic oligoribonucleotides were synthesized for use in GST pull down assays:

21-mer wild-type E2F-1-specific oligoribonucleotide (E-RNA^{wt}) (12):

5'-UAA.GUA.GGA.CGG.AGG.UGG.UCG-3'

21-mer mutant E2F-1-specific oligoribonucleotide (E-RNA^{mut}):

5'-UAA.GUA.GGA.AAU.CUU.AAC.UCG-3'

24-mer wild-type Purα oligoribonucleotide (P-RNA^{wt}) (36):

5'-UCA.GAG.GGC.CUG.UCU.UUC.AAG.GUG-3'

24-mer mutant Purα oligoribonucleotide (P-RNA^{mut})

5'-UCA.GAU.UGC.CUG.UCU.UUG.AAU.UUG-3'

The plasmids pCDNA-E-RNA and pCDNA-P-RNA were constructed as follows. For each plasmid, oligodeoxyribonucleotides were synthesized that contained tandem duplications of the wild-type RNA sequence given above (with T replacing U) and flanking EcoRI sites. The antisense of these oligos were made, annealed, cut with EcoRI and were ligated into the EcoRI site of pCDNA3. Constructs were chosen that had three tandem fragments ligated into the EcoRI site (*i.e.* six copies of the binding sequence). The resulting plasmids (pCDNA-E-RNA and pCDNA-P-RNA) were used to generate E-RNA and P-RNA *in vitro* by transcription from the T7 promoter or to express E-RNA and P-RNA in transfected cells from the CMV promoter.

Cell culture and transfection. U-87MG human glioblastoma cells and J3671 human medulloblastoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco BRL, Rockville, MD, USA) and antibiotics (100 units/ml penicillin, and 10 μg/ml streptomycin). Cells were maintained at 37˚C in a humidified atmosphere containing 7% CO_2. Transfections were carried out by the calcium phosphate precipitation method (37). Briefly, 3 x 10^5 cells were plated on a 60-mm plate and grown overnight. Four hours prior to transfection, the cells were supplied with fresh media and transfection was carried out with 2 μg of the reporter plasmid DHFR-Luc alone or in combination with various expression plasmids including pCDNA-E2F1 (2.5 μg), pCDNA-Purα (2.5 μg or 10 μg), pCDNA-Purα-mutants (2.5 μg or 10 μg). The total amount of DNA in each transfection mixture was kept constant by the addition of empty vector plasmid. Thirty-six hours post-transfection, protein extracts were prepared and a luciferase assay was performed using the Promega Luciferase Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Quantitation was with a luminometer (Femptomaster FB12, Zylux Corporation).

Colony formation assays. Approximately 3 x 10^5 cells were plated in 60-mm dishes and after 24 h, cells were transfected with various expression plasmids (as detailed in the Figure Legends). After 24 h, transfected and untransfected control cells were collected and seeded in 100-mm dishes (5 x 10^4 cells/dish in triplicate) with 0.8 mM G418 alone (cells transfected with pCDNA3-based plasmids) or with 0.8 mM G418 plus 0.15 mM hygromycin (cells cotransfected with pEBV-Purα plasmid). Plates with antibiotics had media replaced with fresh media containing antibiotics every second day. Cells were maintained for 3 weeks, after which, the numbers of colonies were determined by staining of cells with 1% methylene blue for 5 min. For each experiment, the counts from triplicate plates were averaged and standard errors calculated.

In vitro transcription/translation and GST pull-down assay. [^{35}S]-labeled Purα and E2F-1 were synthesized *in vitro* from linearized pCDNA-Purα and pCDNA-E2F-1 using TNT-coupled transcription-translation wheat germ extract (Promega) according to manufacturer's directions. GST-fusion proteins were expressed and purified as described previously (27). Briefly, bacteria were grown, induced for 2 h at 37˚C with 0.5 mM isopropyl-β-D-thiogalactopyranoside, and lysed by sonication. The bacterial lysate was incubated with glutathione-sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and binding of the GST-fusion proteins was allowed to occur overnight at 4˚C. Beads were pelleted and washed. The integrity and purity of the GST-fusion proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining.

For protein-protein interaction studies, approximately 3 μl of [^{35}S]-labeled *in vitro* translated protein (Purα or E2F-1) was incubated with 5 μg of GST or GST-fusion protein immobilized on glutathione-sepharose beads in 300 μl of buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% Nonidet P-40, 1 μg/μl bovine serum albumin) for 2 h at 4˚C with continuous rocking. After the incubation, the beads were pelleted and washed four times with buffer. Bound proteins were eluted with Laemmli sample buffer, heated at 95˚C for 10 min, separated by SDS-PAGE and analyzed by autoradiography. One-tenth of the amount that was used as input for each reaction was loaded as a migration control. For pull-down assays where RNA was used, the RNA was added for 15 min on ice prior to the 2 h incubation with GST-fusion protein.

RNA-protein binding assay (band shift). Binding reactions with purified GST-Purα or GST-E2F-1 were performed in 12 mM Hepes (pH 7.9), 4 mM Tris-HCl (pH 7.5), 60 mM KCl, 5 mM $MgCl_2$, 0.8 mM dithiothreitol and 0.5 μg of poly dI-dC. RNA-protein complexes were allowed to form on ice during a 30-min incubation of the protein sample with 54,000 cpm RNA probe generated from *in vitro* transcription with T7 RNA polymerase of pCDNA-P-RNA or pCDNA-E-RNA (P-RNA and E-RNA) and labeled with polynucleotide kinase and γ-[^{32}P]ATP. Reaction products were analyzed on a 6% polyacrylamide/0.5 x TBE gel and detected by autoradiography.

Results

Purα interacts with E2F-1 in vitro and the E2F-1 binding site localizes to the N-terminus of the Purα protein. Our previous studies had shown that Purα and E2F-1 directly bind to each other (23). In order to further investigate this phenomenon, we sought to determine the region within Purα that is involved in E2F-1 binding. *In vitro* translated radiolabeled E2F-1 was utilized in GST pull-down assays with full length Purα (1-322) and various Purα deletion mutants. Figure 1A shows that full-length Purα bound to E2F-1 (lane 3), but the N-terminal deletion mutants of this protein that progressively removed the residues between 1 to 167, 1 to 216 and 1 to 274 showed no ability to interact with E2F-1 (lanes 4-6, respectively). This indicates that the N-terminal 167 amino acids of Purα are required for E2F-1 interaction.

Figure 1B shows an experiment in which Purα C-terminal deletion mutants were examined. Interestingly the deletion mutant lacking the region between residues 216-322 (mutant 1-215) showed even greater binding to E2F-1 than full-length Purα (compare lanes 4 to lane 3 in Figure 1B). This suggests that, not only is the E2F-1 binding site in the N-terminus of Purα, but also that the Purα C-terminus may actually partially interfere with E2F-1 binding.

Further deletion from the C-terminus of Purα that removed the residues between 174 to 322 (mutant 1-174) decreased binding, suggesting a potential enhancing domain between amino acid residues 174 and 215. Further deletion including 154 to 322 (1-154) and 123 to 322 (mutant 1-123) had no effect on Purα interaction with E2F-1 (compare lanes 6-7 to lane 5 in Figure 1B). The largest C-terminal deletion tested was one that left only 72 amino acids of Purα (mutant 1-72) and this showed a modest decrease in the interaction between Purα and E2F-1 (lane 8).

Figure 1C shows the remaining Purα deletion mutants that were designed to test the importance of central regions of Purα. As expected, mutants lacking the N-terminus showed very little binding of E2F-1 (lanes 5-7). Interestingly Purα mutant Δ72-231 (in which a central region has been deleted and the N- and C- termini of Purα fused) has very little binding of E2F-1. This might indicate that residues in the deleted central domain are important for E2F-1 binding to occur or alternatively that the C-terminus of Purα may interfere with E2F-1 binding as was suggested above.

A summary of the experimental results pertaining to the regions of Purα that are important for E2F-1 binding is shown in Figure 1D. A schematic diagram of full-length Purα is presented showing the known domains and motifs (adapted from our recent review (20)). The structure of the Purα deletion mutants that were used are shown and the results obtained with them are indicated. These results, summarized on the right, demonstrate that the N-terminal region of Purα, specifically amino acids 1 to 72, comprises the E2F-1 binding domain.

Purα expression inhibits E2F-1-mediated transcriptional activation of the DHFR promoter and this function maps to the N-terminus of Purα. U-87MG cells were transfected with combinations of the following plasmids and then the luciferase

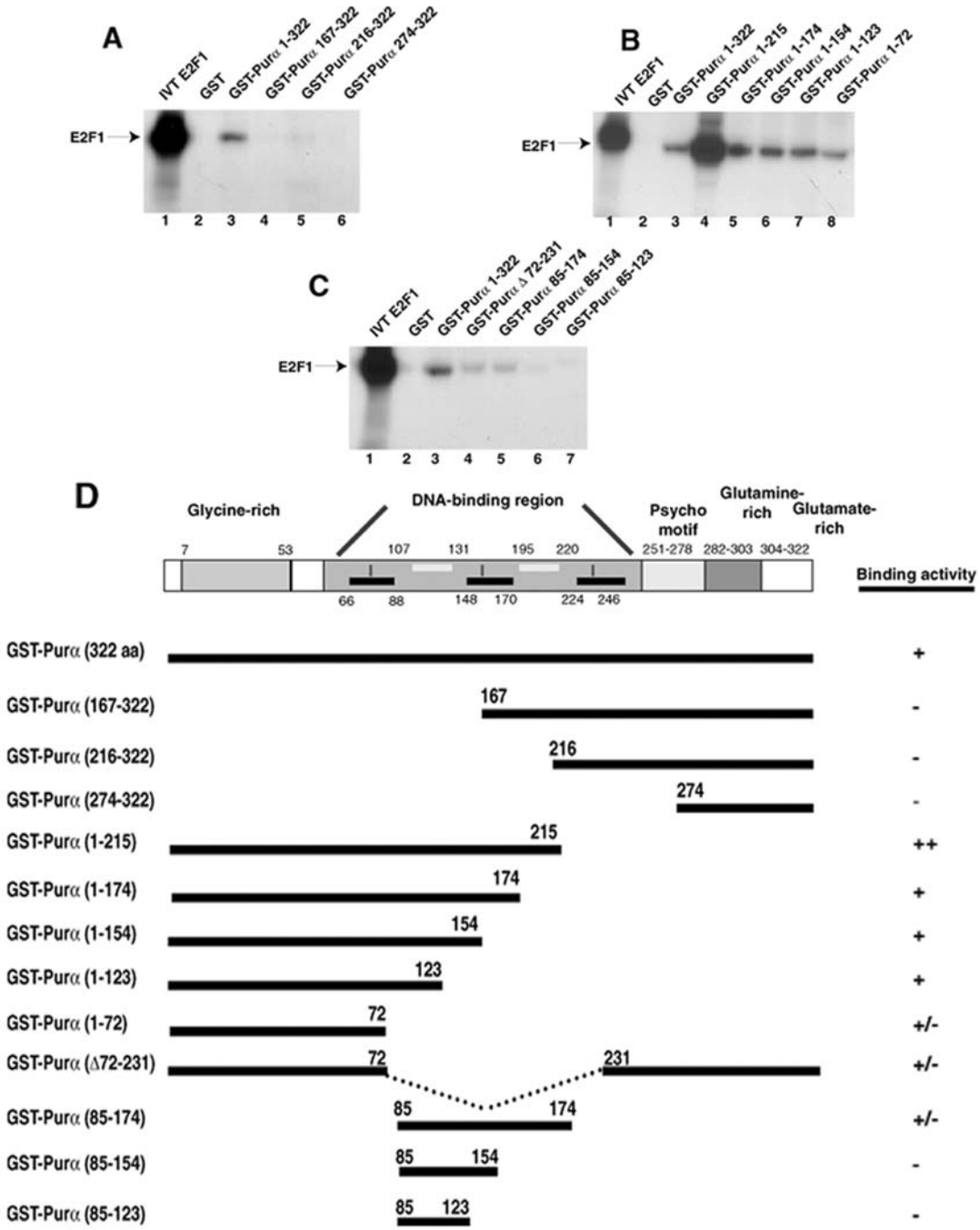

Figure 1. *Interaction of Purα with E2F-1 and localization of E2F-1 binding site within Purα. GST-pull-down assays were performed by using in vitro synthesized, [^{35}S]-labeled E2F-1 incubated with the following: Panel A - GST-Purα N-terminal deletion mutants; Panel B - GST-Purα N-terminal deletion mutants; Panel C - GST-Purα central region deletion mutants on beads. An arrow shows the position of the E2F-1 band in each panel. A. In vitro translated, [^{35}S]-labeled E2F-1 was incubated with GST (lane 2) or GST-Purα fusion protein (lane 3) or GST-Purα N-terminal deletion mutants (lanes 4-6) immobilized on glutathione-sepharose beads and bound protein separated by SDS-PAGE and detected by autoradiography. The GST fusion proteins used are indicated above each lane with the numbers of the amino-acid residues at their N-terminus and their C-terminus (full length Purα is GST-Purα1-322). An amount equivalent to 10% of the input labeled E2F-1 protein used for the assay was run as a positive control (lane 1). B. As for Panel A except with various C-terminal deletion mutants of Purα. GST (lane 2), GST-Purα (lane 3) and GST-Purα C-terminal deletion mutants (lanes 4-8) as indicated. Lane 1 contains 10% of the input amount of [^{35}S]-labeled E2F-1. C. As for Panels A & B except with GST-Purα central region mutants (lanes 4-7). D. A summary of the experimental results pertaining to the regions of Purα that are important for E2F-1 binding is shown. A schematic diagram of full-length Purα is shown with known domains and motifs indicated. This is adapted from our recent review (20). The structures of the Purα deletion mutants used are shown together with a summary of the results obtained with them in the experiments performed in Panels A-C.*

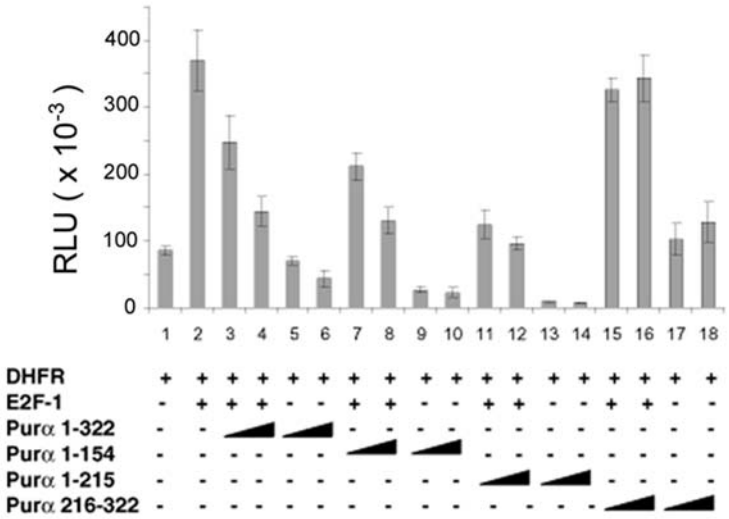

Figure 2. *Effect of Purα and Purα deletion mutants on E2F-1-mediated transcriptional activation of the DHFR promoter. U-87MG cells were transfected with 2 μg of DHFR-Luc. Parallel cultures were transfected with the DHFR-Luc plasmid with 2.5 μg pCDNA-E2F-1 and/or pCDNA-Purα and pCDNA-Purα deletion mutants. The plasmids pCDNA-Purα or pCDNA-Purα mutants were used at low (2.5 μg) and high (10 μg) DNA concentrations indicated by the ramp symbol (i.e., 2.5 μg ◢ 10 μg). After 36 h, protein extracts were prepared and luciferase activity assayed. The luciferase activity expressed by the DHFR promoter in the presence (lanes 2-18) and absence (lane 1) of each protein is an average of at least 2 experiments with a standard deviation ranging from ± 10%-20%.*

activity expressed in cell extracts was assayed (Figure 2): (i) DHFR-Luc; (ii) pCDNA-E2F-1; and (iii) pCDNA-Purα or pCDNA-Purα deletion mutants. The plasmid combinations used are indicated in the lower part of the figure. The plasmids pCDNA-Purα or pCDNA-Purα deletion mutants were used at low (2.5 mg) and high (10 mg) DNA concentrations and this indicated by the ramp symbol (◢). The luciferase activity expressed by the DHFR promoter in each transfection (lanes 1-18) is an average of at least 2 experiments with a standard deviation ranging from ±10%-20% (shown as an error bar). In the presence of plasmid expressing E2F-1, the level of transcription from DHFR promoter was increased (compare lane 1 to lane 2, Figure 2). In contrast, ectopic expression of Purα in U-87MG cells transfected with DHFR-Luc resulted in the inhibition of the DHFR promoter in these cells (Figure 2, lanes 5 and 6). Co-expression of E2F-1 and Purα in the cells caused decrease in the level of E2F-1-mediated transactivation of the DHFR promoter (compare lanes 3 & 4 with lane 2, Figure 2). This has also been observed with the E2F promoter (Darbinian, unpublished data).

The effect of three Purα deletion mutants (1-154, 1-215 and 215-322) on the transcriptional activity of DHFR promoter was examined. Co-expression of E2F-1 and Purα1-154 or E2F-1 and Purα1-215 caused a decrease in the level of E2F-1-induced transcription of the DHFR promoter (Figure 2, lanes 7-8 and 11-12, respectively). However, deletion mutant Purα216-322 did not suppress E2F-1-activated DHFR transcriptional activity (lanes 15-16). Thus the inhibition activity of Purα mutants in the E2F-1-activated DHFR promoter assay correlated with their binding activity as measured in Figure 1. These results support the conclusion that suppression of the action of E2F-1 on the DHFR promoter by Purα is mediated through the interaction between Purα and E2F-1.

The integrity of each of the mutant Purα proteins within the transfected cells was determined by Western blot analysis (data not shown).

The inhibition of cell growth by E2F-1 in U-87MG cells is reversed by Purα and Purα deletion mutants. Colony formation by human U-87MG glioblastoma cells was measured after transfection with E2F-1 and Purα expression plasmids. In Figure 3A, U-87MG cells were transfected with pCDNA3 (control vector), pCDNA-Purα, pCDNA-E2F-1, or pCDNA-Purα plus pCDNA-E2F-1. Colonies that survived in the presence of G418 were counted after methylene blue staining as described in Materials and Methods. Ectopic expression of Purα alone gave an 80% inhibition of colony formation compared to vector control (compare plates 1 and 2 in Figure 3A) in agreement with our previous observations regarding growth inhibitory effects of Purα on U-87MG cells (31). Similarly, ectopic expression of E2F-1 alone gave a 79% inhibition of colony formation (compare plates 1 and 3 in Figure 3A). Growth inhibition by ectopic expression by E2F-1 has been reported in many cell types and is thought to be due to activation of p53 (6-9). However when Purα and E2F-1 were co-expressed, only a 29% inhibition of cell growth was observed (compare plates 1 and 4 in Figure 3A). In other words, the growth inhibitory effect of each protein was partially reversed when both proteins were overexpressed.

In order to localize the region of Purα that is important for suppression of E2F-1-associated cell growth inhibition, the ability of full-length Purα and three Purα deletion mutants

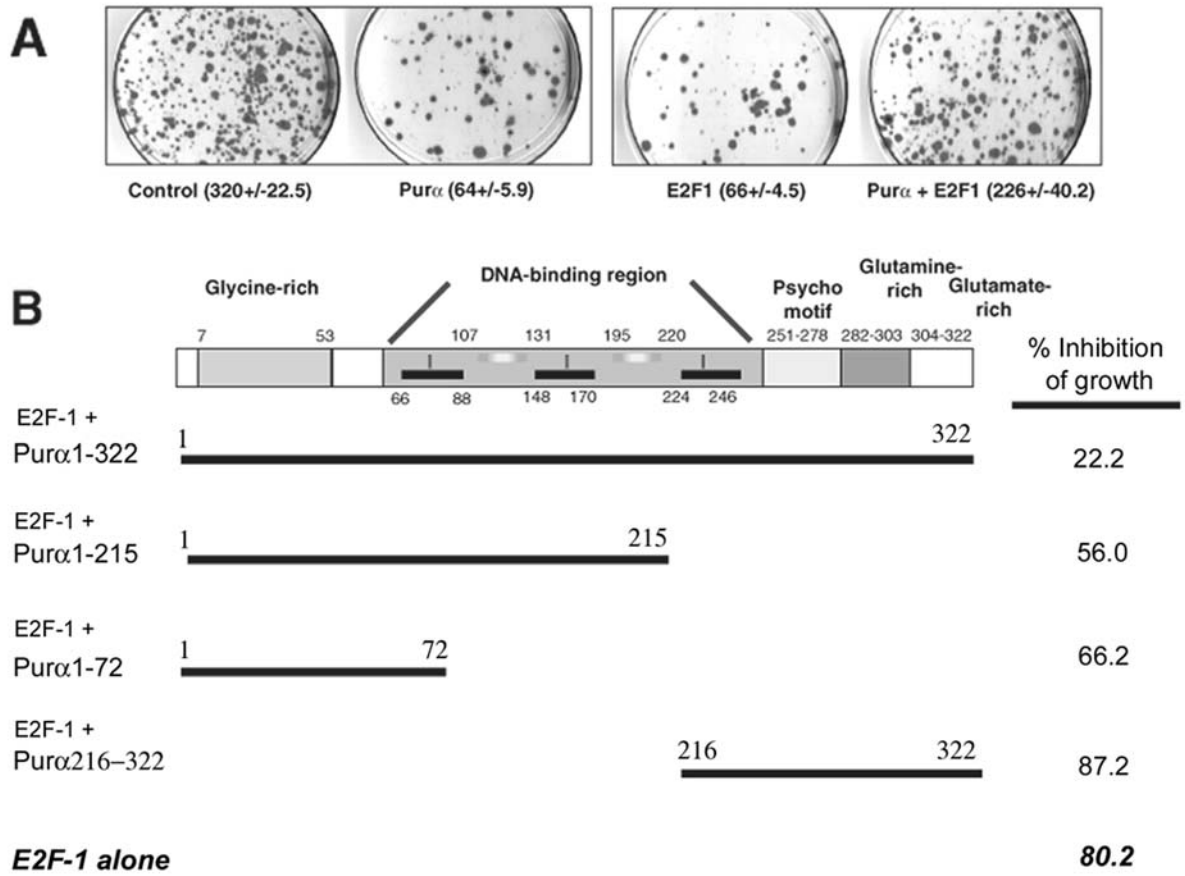

Figure 3. *Effect of E2F-1 and Purα on the growth of U-87MG cells. Colony formation by U-87MG cells was measured after transfection with Purα and E2F-1 expression plasmids. A. U-87MG cells were transfected with pCDNA3 vector control, pCDNA-Purα, pCDNA-E2F-1, or pCDNA-Purα plus pCDNA-E2F-1. The number of colonies that survived in the presence of G418 was determined after 3 weeks as described in Materials and Methods. The numbers represent the average of two independent experiments ± the standard deviation. B. A schematic representation of the structure of Purα is shown. Below are shown the 322 amino acid full-length Purα and three Purα deletion mutants (1-215, 1-72 and 216-322) that were used in colony formation assays. The ability of full-length Purα and these mutants in the suppression of E2F-1-associated U-87MG cell growth inhibition was examined in colony formation assays as described in panel A except that pEBV-Purα (full-length and deletion mutants) was used and selection was with hygromycin plus G418. The percent inhibition of U-87MG cell growth upon expression of E2F-1 with full-length and various mutants of Purα was determined and is shown in the right-hand column. The percent inhibition represents the average of two independent experiments ± the standard deviation.*

(1-215, 1-72 and 216-322) were used in the colony formation assay with E2F-1 (Figure 3B). In the presence of overexpression of E2F-1, full-length Purα gave a 22% inhibition of growth, *i.e.* it strongly reversed E2F-1-associated growth inhibition consistent with results in Figure 3A (29%). The C-terminus of Purα was ineffective in this assay resulting in 87% inhibition of colony formation. The deletion mutants of Purα that retained the N-terminus (1-71 and 1-215) were only partially effective in reversing the inhibition of growth (66% and 56%, respectively). This suggests that interactions of Purα, other that its N-terminal binding to E2F-1, may also be important for reversing the inhibition of growth caused by E2F-1. Although Purα1–215 had increased binding to E2F-1, it had only partial capability in reversing growth inhibition.

In colony formation assays, the plasmids pCDNA-Purα and Purα deletion mutants, which require G418 selection, gave similar results to those obtained with pEBV-Purα and Purα deletion mutants, which require hygromycin selection (data not shown).

Binding of Purα and E2F-1 to P-RNA and E-RNA. Both E2F-1 (12) and Purα (26) have been demonstrated to form sequence-specific associations with RNA molecules. The binding of Purα and E2F-1 to RNA transcripts that contain their respective binding sequences (P-RNA and E-RNA, respectively) was demonstrated in band-shift assays using radiolabeled E-RNA or P-RNA probes generated from *in vitro* transcription with the pCDNA-E-RNA and pCDNA-P-RNA plasmids (Figure 4A

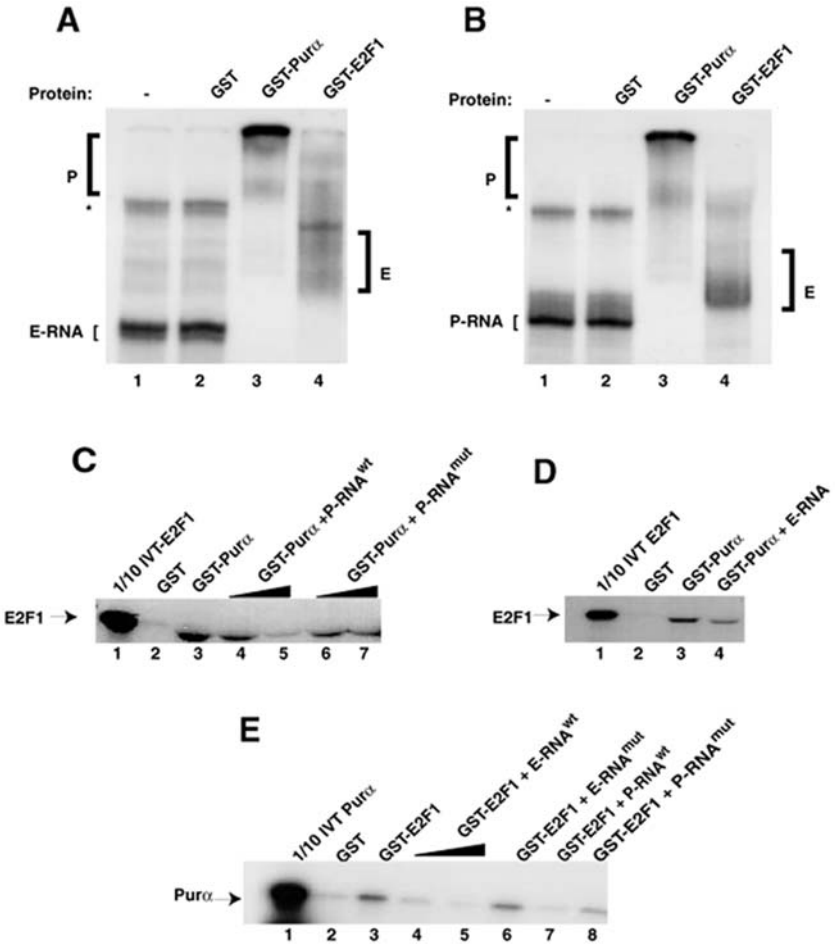

Figure 4. *Binding of Purα and E2F-1 to the P-RNA and E-RNA oligonucleotides. A & B. In vitro transcribed and radiolabeled E-RNA (Panel A, lane1) or P-RNA (Panel B, lane 1) were used as probes in band-shift assays. Probes were incubated with 0.5 μg GST (lanes 2), 0.5 μg GST-Purα (lanes 3) and 0.5 μg GST-E2F-1 (lanes 4). The positions of the probe and the Purα:RNA complex (P) or E2F-1:RNA complex (E) are shown by brackets. Lane 1 represents probe alone. The * indicates a minor transcript that may be due to run-through transcription. C-D. GST-pull-down assays were performed by using in vitro synthesized, [^{35}S]-labeled E2F-1. E2F-1 was incubated with beads carrying GST (lanes 2) or GST-Purα (Panel C, lanes 3-7; Panel D, lanes 3 & 4). Synthetic RNAs (1 μg ◢ 5 μg in Panel C, 1 μg in Panel D) were added as indicated. One-tenth of the input reaction amount of E2F-1 was loaded as a migration control (lane 1). An arrow shows the position of the E2F-1 or Purα band in each panel. E. GST-pull-down assays were performed by using in vitro synthesized, [^{35}S]-labeled Purα. Purα was incubated with beads carrying GST (lane 2) or GST-E2F-1 (lanes 3-8). Synthetic RNAs (1 μg ◢ 5 μg for E-RNAwt; 5 mg for E-RNAmut and P-RNAs) were added as indicated. One-tenth of the input reaction amount of Purα was loaded as a migration control (lane 1).*

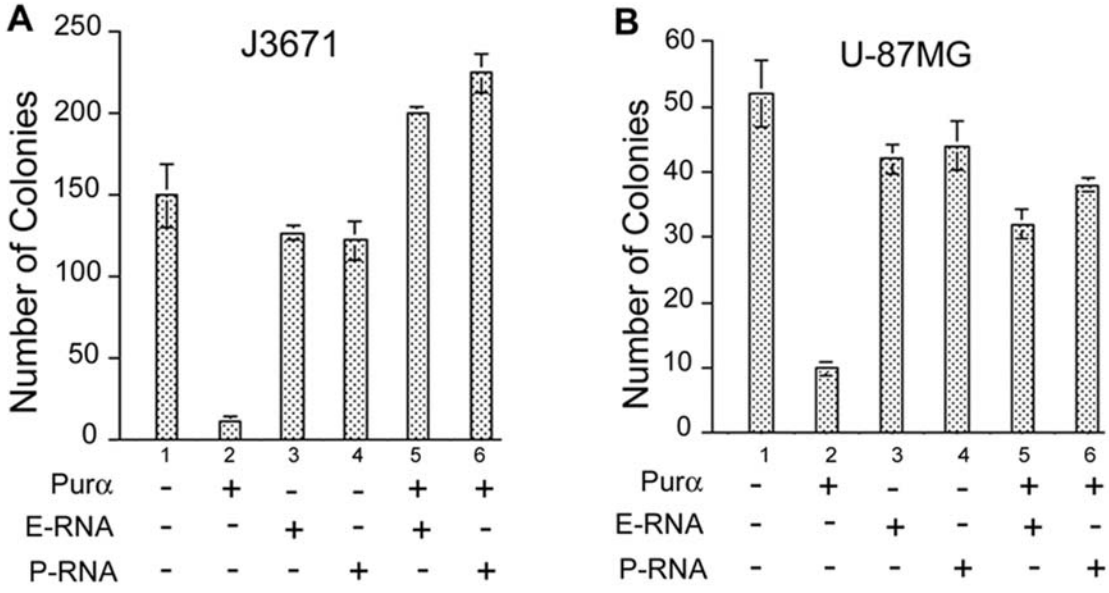

Figure 5. *Effect of E-RNA and P-RNA expression on Purα-mediated suppression of cell growth in J3671 and U-87MG cell lines. Human J3671 medulloblastoma cells (Panel A) and U-87MG glioblastoma cells (Panel B) were transfected with pCDNA3, pCDNA-Purα, pCDNA-E-RNA and pCDNA-P-RNA as indicated, and the number of colonies which survived in the presence of G418 were determined after 3 weeks in colony assay. The columns represent the average of three independent experiments ± the standard deviation. The left-hand column in each panel is the pCDNA3 control.*

and B, respectively). We showed that Purα binds to the Purα-specific P-RNA (Figure 4B, lane 3). Interestingly Purα was also able to bind to E2F-1-specific RNA (Figure 4A, lane 3) which may be due to the presence of GC-rich repeats in the sequence of E-RNA. As for E2F-1, binding of this protein to E-RNA is shown (Figure 4A, lane 4). Also, it was found that E2F-1 bound to P-RNA (Figure 4B, lane 4).

RNA molecules modulate interaction between E2F-1 protein and Purα. GST-pull-down assays were used to investigate the effects of P-RNA and E-RNA on Purα/E2F-1 binding *in vitro*. In these experiments, the synthetic 24-mer P-RNA and 21-mer E-RNA oligoribonucleotides were used (described in Materials and Methods). E2F-1-binding to immobilized Purα was inhibited by wild-type P-RNA (compare lanes 4 & 5 to lane 3 in Figure 4C) but not by P-RNA in which the Purα-binding sequence had been mutated (Figure 4C, lanes 6 & 7). E-RNA also inhibited E2F-1 binding to immobilized Purα (compare lanes 3 and 4 in Figure 4D). Similarly Purα-binding to immobilized E2F-1 was inhibited by E-RNA (compare lanes 4 &5 to lane 3 in Figure 4E) and by P-RNA (lane 7) but not to mutated versions of E-RNA or P-RNA (lanes 6 and 8, respectively).

Suppression of cell growth in J3671 and U-87MG cell lines by Purα is eliminated in the presence of E-RNA and P-RNA. The effect of E-RNA and P-RNA was examined by transfection of J3671 human medulloblastoma cells (Figure 5A) or U-87MG cells (Figure 5B) with pCDNA-E-RNA and pCDNA-P-RNA. Purα markedly suppressed the growth of both J3671 and U-87MG cells. In both cell types, expression of E-RNA or P-RNA alone had little effect on cell growth. However when co-expressed with Purα, E-RNA and P-RNA reversed Purα-associated inhibition of cell growth in both cell types.

Discussion

E2F is a critical regulator of the cell cycle. Many genes whose expression is required for S-phase are up-regulated by E2F (1). Therefore controlling the level of active E2F in the cell is essential for appropriate progression of the cell cycle. Perhaps the best-known mechanism for regulating the level of free active E2F is through its binding and sequestration by pRB. pRB has multiple phosphorylation sites and is thus able to act as an integrator of signals from multiple protein kinase signaling pathways that are activated during G1 progression. Hyperphosphorylation of pRb causes the release of E2F at an appropriate time to allow cells to enter S-phase (2-4). In addition to pRb, E2F-1 can also bind to the protein Purα, an interaction that also inhibits E2F activity (23). We have now found that the binding site for E2F-1 resides in the N-terminus of Purα and this is also the site that blocks the biological activity of E2F-1 as measured by its ability to transactivate the DHFR promoter. The enzyme DHFR is required for the biosynthesis of nucleotides during the S-phase of the cell cycle and the sensitivity of the promoter of the DHFR gene to E2F-1 makes DHFR promoter transactivation a sensitive readout of E2F-1 activity. Since the same region of Purα is involved in both binding and inhibition of E2F-1, it seems likely that it is the binding of Purα to E2F-1 that causes its inhibition. Interestingly E-RNA prevents the binding of Purα to E2F-1. This RNA was isolated from an RNA library by its ability to bind to E2F-1 (12). The library was prescreened with a mutant E2F-1 in which the DNA-binding domain was mutated so that RNAs would be selected that only bind to the E2F-1 DNA-binding domain. Indeed E-RNA prevents E2F-1 binding to its recognition sequence in double-stranded DNA in band-shift assays (12). Taken together, these data suggest that the E2F-1 binding site for E-RNA and Purα corresponds to its DNA-binding domain and thus the binding of Purα to E2F-1 may inhibit E2F-1 function by blocking the ability of E2F-1 to bind to DNA. We previously demonstrated that Purα inhibits E2F-1 binding to the DHFR binding site (23).

Although E2F-1 promotes cell cycle progression, ectopic expression of E2F-1 inhibits cell proliferation and this is thought to be due to the activation of the p53 checkpoint mechanism (6-9). Presumably this is a defense mechanism against abnormal cell proliferation occasioned by E2F activation that might occur if pRb was inappropriately deactivated. This might occur through pRb hyper-phosphorylation due to activation of an oncogenic protein kinase pathway, mutational loss of the RB1 gene or binding of pRB by a viral transforming protein (10). Clearly U-87MG cells are growth-inhibited by ectopic E2F-1 expression and this is reversed by co-expression of Purα (Figure 3). The simplest explanation of this would be that Purα binds to E2F-1 and reduces the growth-inhibitory supraphysiological concentration of E2F-1 found in the E2F-1-transfected cells. However, the Purα deletion mutants that retain the N-terminus (1-72 and 1-215) bind E2F-1 well but are only partially active in the rescue of cells from E2F-1-associated growth inhibition. Therefore the situation must be more complex and involve functions pertaining to the central domain of Purα. Such functions might include the activity of Purα as a transcription factor and/or binding of Purα to other proteins, *e.g.* pRb.

The interaction of Purα with E2F-1 is a double-edged sword since overexpression of Purα alone is growth inhibitory. So it is also valid to interpret the data from the viewpoint that E2F-1 can rescue cells from the growth inhibitory effects of ectopic Purα expression. Interestingly the E-RNA and P-RNA species (which bind to Purα and prevent it from associating with E2F-1) are also able to

reverse the growth-inhibitory effects of ectopic Purα overexpression. While E-RNA is an artificial RNA derived from a library (12), P-RNA is based on sequences known to exist in U-87MG cells based on RT-PCR and sequence analysis of RNA that co-immunoprecipitated with Purα from U-87MG nuclear extracts (26,36). Thus it is likely that regulation of the activities of Purα by RNA is a physiologically relevant event.

Recently we have reported the creation of transgenic mice with inactivation of the PURA gene that encodes Purα, revealed that Purα has an essential role in postnatal brain development (38). PURA$^{-/-}$ mice appear normal at birth, but at two weeks of age, they develop neurological problems and they die by four 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. It will now be possible to explore E2F-1 function in a cellular environment lacking Purα using embryo fibroblasts from these PURA$^{-/-}$ mice.

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