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Optimizing Aptamer Activity for Gene Therapy Applications Using Expression Cassette SELEX

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RNA aptamers against a variety of clinically relevant target proteins have been generated. For example, we previously isolated an RNA aptamer that inhibits the function of the E2F family of transcription factors that play a critical role in the control of cell proliferation. However, the development of this and other aptamers for gene therapy applications has been complicated by the fact that expression of RNA aptamers in the context of flanking sequences can inhibit the ability of an aptamer to fold into its functional conformation. Insertion of the E2F aptamer into a tRNA expression cassette resulted in the production of high levels of chimeric tRNA that contains a misfolded and inactive aptamer in transfected mammalian cells. To overcome this problem, we randomized the sequence flanking the aptamer and selected for chimeric tRNAs that retained high affinity binding to E2F1. This expression cassette SELEX strategy yielded RNAs that bind E2F with high affinity (IC_{50} of 15nM) and which can be expressed at high levels in mammalian cells. Moreover, these chimeric tRNA-E2F aptamers are functional and can inhibit E2F-mediated transactivation by up to 80% in human 293 cells. Expression cassette SELEX should greatly facilitate the use of aptamers for a variety of gene therapy applications.

Key Words: gene therapy, E2F, aptamer, SELEX

INTRODUCTION

Many gene therapy applications have been envisioned for decoy RNAs, RNA aptamers, and ribozymes. Such RNAs can adopt structures that allow them to bind and inhibit the activity of target proteins (decoys and aptamers) [1] or cleave and destroy or splice and repair target transcripts (ribozymes) [2,3]. The use of these structured RNAs in gene therapy applications is complicated by the fact that therapeutic RNA sequences are usually expressed as part of longer transcripts and flanking RNA sequences can interfere with the ability of therapeutic RNA to adopt their functional conformations.

Because the E2F family of transcription factors plays a critical role in the control of cell proliferation, DNA decoys [4–6] and RNA aptamers [7] that target these proteins to combat hyperproliferative processes have been generated. That the E2F transcription factors are critical for the normal process of cell proliferation [8,9] is evident from experiments using over-expression of E2F proteins to drive cell cycle progression [10] as well as experiments that demonstrate an impaired proliferative capacity in cells deficient in E2F activities [11,12]. Moreover, the deregulation of E2F activity that results from mutations in various components of the Rb pathway is tightly associated with oncogenic processes [13,14]. These observations suggest that the inhibition of E2F activity would be an effective means to block cell proliferation and thus would be an attractive therapeutic target for hyper-proliferative processes such as vascular restenosis and cancer. Indeed, others have shown that inhibition of E2F in vascular and renal settings can inhibit pathologic tissue proliferation [4,5,15,16].

We have previously used *in vitro* selection to identify RNA aptamer sequences that bind with high affinity to E2F proteins and that have the capacity to inhibit E2F DNA binding activity [7]. Moreover, one E2F aptamer, termed E2F-E1, also was shown to inhibit cell cycle re-entry when microinjected into quiescent cells that were stimulated to grow [7]. This latter result is consistent with the critical role of E2F activities in controlling the G1/S phase transition during a growth stimulatory response.

Although this work established the potential utility of an RNA aptamer for controlling E2F function and cellular proliferation, it was nevertheless limited by the fact that the RNA could only be shown to function *in vivo* by

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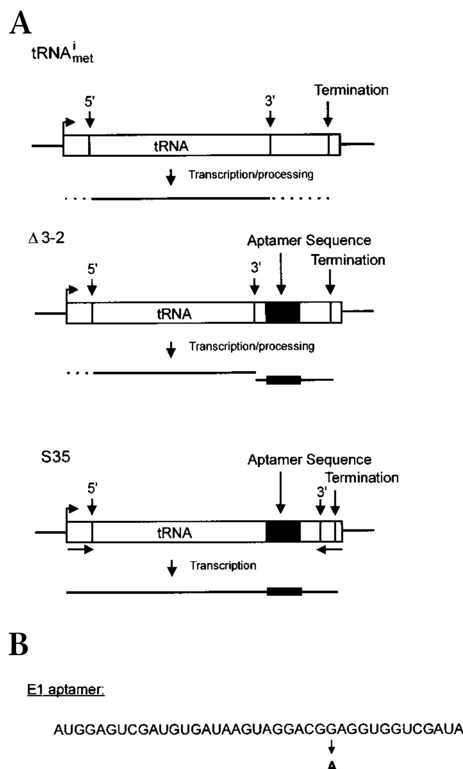

FIG. 1. Schematic diagram of expression constructs. (A) Derivation of the S35 expression vector. The S35 and Δ3-2 constructs are based on a modified $tRNA_{met}$ gene that contains an internal promoter. The Δ3-2 vector allows for insertion of the aptamer sequence following the 3′ processing signal. Thus, the aptamer sequence would be included in the 3′ fragment generated after RNA processing of the tRNA transcript. The S35 vector places the aptamer sequence before the 3′ processing signal. In addition, this vector also contains a sequence encompassing the termination site that is complementary to the 5′ end of the transcript (indicated by the arrows). As such, this allows the transcript to form a hairpin involving these sequences and also prevents processing of the RNA at the 5′ and 3′ sites. Thus, the aptamer sequence is contained within an extended transcript of greater stability. (B) Sequence of the previously identified E2F-E1 aptamer that is selective for E2F proteins [7]. The arrow indicates the change of G to A in the S4.2m mutant version of the aptamer.

microinjection. Here we describe the development of an expression cassette for the E2F-E1 aptamer that allows that RNA to retain high-affinity binding to E2F and to be expressed at high levels in mammalian cells. To generate this functional expression cassette, we used *in vitro* selection methods to identify a sequence context that did not significantly interfere with the proper folding of the E2F-E1 aptamer. This selection approach, termed expression cassette SELEX, should facilitate the development of other structured RNAs for gene therapy applications.

RESULTS

Constructs for *in Vivo* Expression of RNA Aptamers

Our previous work described the use of the SELEX procedure to isolate RNA aptamers that bound to E2F proteins with high-affinity [7]. One such RNA molecule, termed E2F-E1, was shown to exhibit high-affinity binding to E2F proteins leading to an inhibition of E2F DNA-binding activity. Moreover, the same RNA, when microinjected into growth stimulated fibroblasts, blocked the ability of the cells to enter S phase. Although these experiments demonstrate the potential of an RNA aptamer as an inhibitor of E2F activity and cell proliferation, they fall short of suggesting a practical approach to the use of this sequence because of the limitations of RNA delivery to cells. As such, we have now explored the ability of the E2F-E1 aptamer sequence to inhibit E2F function when expressed as part of a larger RNA transcript within mammalian cells.

We initially evaluated several expression systems to produce the E2F-E1 aptamer sequence *in vivo*. Attempts to use RNA polymerase II-based systems were unsuccessful due to the low level of RNA accumulation in transfected cells (data not shown). We then turned to RNA polymerase III (pol III)-based systems in light of previous experience that indicated that such promoters can produce substantial levels of RNA in cells. Figure 1 shows a schematic diagram of the Δ3-2 RNA polymerase III construct that was derived from a human methionine tRNA gene that has been used to express antisense and decoy RNAs in cell lines that are stably transduced with a retroviral vector termed DCt5T′ [17,18]. The S35 cassette (Fig. 1) was modified to produce a stable intramolecular duplex between the 5′ and 3′ termini, which resulted in enhanced accumulation of the product [19].

A Strategy for the *in Vivo* Expression of Previously Selected RNA Aptamers

As an initial step to assay the *in vivo* inhibitory capacity of the S35-expressed E2F aptamer, we generated RNA transcripts *in vitro* to measure their effectiveness in binding to and inhibiting activity of E2F. In particular, it was important to determine to what extent the context of the tRNA molecule affected the function of E2F-E1. A T7 promoter was placed at the 5′ end of the S35-E1 sequence such that, when transcribed *in vitro* with T7 RNA polymerase, the resulting primary sequence was identical to that when S35-E1 was transcribed *in vivo* by way of the pol III promoter. This system was used to generate RNA *in vitro* to assess its ability to bind to and inhibit E2F DNA-binding activity. Although the original E2F-E1 RNA sequence was effective at inhibition of E2F1 DNA binding, the IC_{50} for the *in vitro* transcribed S35-E1 to inhibit E2F DNA binding was substantially higher (approximately 200 nM; data not shown). Thus, it appears that placing the E2F-E1 RNA sequence in the context of the tRNA molecule altered its ability to bind the E2F1 protein.

In an attempt to alleviate the negative impact of the S35 context, the S35-E1 molecule was modified by inserting 10 random bases on either side of the E2F-E1 stem-loop insert. The RNA pool was then subjected to additional rounds of selection for E2F1 binding. One of the resulting clones, S4.2, is shown in Fig. 2A with a proposed secondary structure determined by the MulFold program [20]. S4.2 inhibited E2F1 binding to the DHFR promoter which contains E2F recognition sites with an IC_{50} of approximately 15 nM (Fig. 2B), in sharp contrast to the binding of the parent RNA, which has an IC_{50} of 200 nM (S35-E1). The binding of S4.2 to E2F1 is still reduced from that of the original E2F-E1 E2F aptamer, which binds with a K_d of 2–4 nM, but it is significantly stronger than that of the

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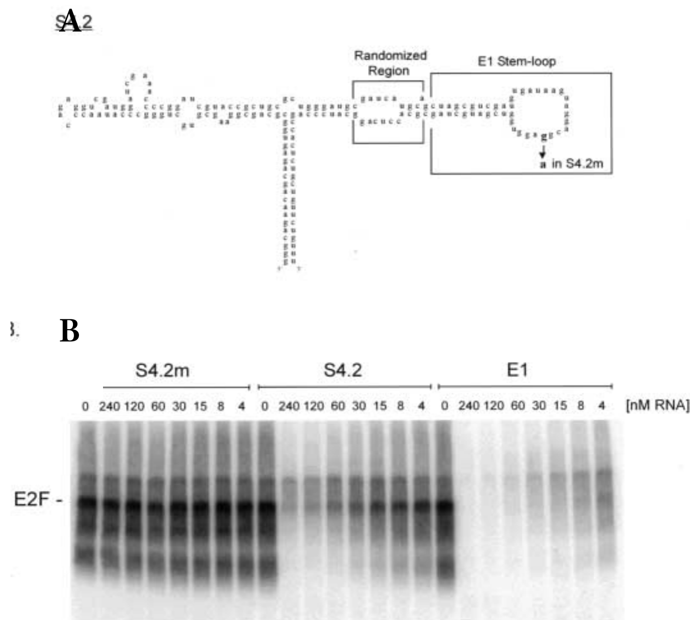

FIG. 2. *In vitro* inhibition of E2F activity. (A) Structure of the S4.2 RNA. The secondary structure of S4.2 was derived using the MulFold program [20]. The randomized region represents the 20 bases that were randomized before further selection. (B) GST-E2F1 was bound to a ^{32}P-labeled adenovirus E2 promoter fragment in the presence of varying concentrations of *in vitro*-transcribed S4.2m, S4.2 and the native E2F-E1 molecule. Complexes were separated on an acrylamide gel and autoradiographed.

initial tRNA construct. In addition, we also constructed a mutant of S4.2 (S4.2m) patterned after the mutant for E2F-E1 that disrupted E2F binding [7]. This same mutation, located in a predicted loop structure, essentially abolished the binding of S4.2 to E2F1 (Fig. 2B).

Inhibition of E2F Activity by Expression of RNA Inhibitors

The ability of the pol III based constructs to direct significant RNA accumulation in cells, together with the ability of the modified transcripts to inhibit E2F DNA binding activity *in vitro,* represents the first critical steps in developing an *in vivo* inhibitor of E2F activity. However, the mere production of an RNA that is capable of inhibiting E2F binding *in vitro* does not guarantee that it will be effective when expressed within the cell. Various unknown events need to occur for the RNA to function in the desired manner, for example, achieving an appropriate intracellular localization or folding in a manner that would allow an interaction with E2F proteins despite the milieu of RNA binding proteins within the cell. Thus, to determine if the RNA that is capable of inhibiting E2F activity *in vitro* is also functional within a cell, we developed an assay to measure the effect of the S4.2 RNA on E2F activity *in vivo*.

The S35 construct generated significant accumulation of the RNA aptamer product (Fig. 3), whereas the Δ3-2 vector was only poorly expressed. We estimate that approximately 5×10^5 to 1×10^6 molecules of S35-derived RNA aptamer were produced per cell. In contrast, the accumulation of transcripts from a polymerase II expression cassette was below the limits of our detection (data not shown). Moreover, our choice of expression cassette was dictated by previous observations that the chimeric tRNAs tend to accumulate in the nucleus [21,22], which is likely to be an important requirement for the inhibition of a nuclear transcription factor.

We assessed the capacity of a transfected plasmid encoding the S4.2 chimeric tRNA transcript to affect E2F activity *in vivo*. As an assay for E2F activity, 293 cells were transfected with an E2-*CAT* (chloramphenicol acetyl transferase) plasmid in which the *CAT* gene is controlled by the E2F-dependent adenovirus E2 promoter. The cells were co-transfected with plasmids encoding E2F1 and DP1, the heterodimer partners that create E2F1 activity, resulting in approximately 10-fold stimulation of CAT activity. Cotransfection of the plasmid containing the S4.2 construct led to an inhibition of E2F activity in a dose-dependent fashion, whereas cotransfection of the plasmid encoding the S4.2 mutant had no effect on the activity of the E2F-dependent activation of the promoter (Fig. 4). Thus, the efficient accumulation of an E2F inhibitory RNA does indeed have the capacity to inhibit E2F activity within mammalian cells if the aptamer sequence is presented in the proper context.

DISCUSSION

The rapid advances in gene discovery, together with the development of knowledge about gene function and pathways of gene function in relation to disease processes, have provided the opportunity to develop gene-targeted therapies. Although the isolation of small-molecule inhibitors to such proteins remains a viable and effective strategy to the development of therapeutic molecules, an alternative

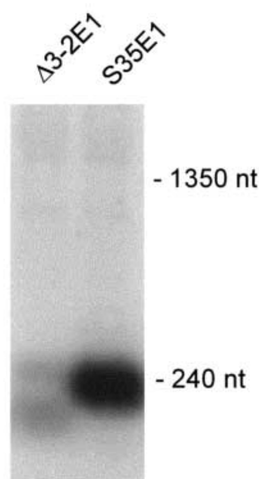

FIG. 3. Intracellular expression of E2F inhibitors. Plasmids containing the Δ3-2-E1 and S35-E1 sequences were transfected into 293 cells by calcium phosphate precipitation. Cells were lysed and RNA was isolated 24 hours after transfection. Total RNA was separated on a denaturing gel, blotted onto a nylon membrane and probed with a labeled anti-E2F-E1 oligonucleotide. RNA size markers are shown at the right.

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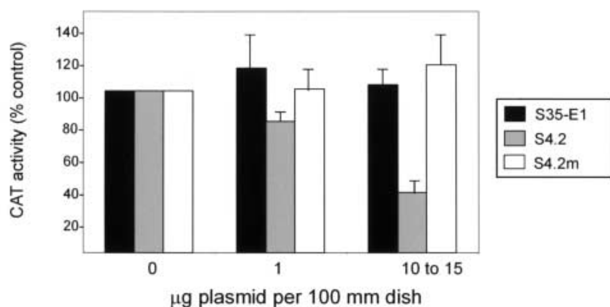

FIG. 4. Inhibition of E2F-mediated transactivation *in vivo*. This figure is a compilation of several independent experiments comparing S4.2, S4.2m, and S35-E1. Values have been normalized to dishes treated with equal microgram quantities of control plasmid. Error bars represent the standard error of the mean.

approach uses the ability to carry out selections for small RNA molecules that can bind with high affinity to target proteins. SELEX technology has resulted in the identification of several small RNA molecules that bind with high affinity to a target protein [23,24,24–28]. Nevertheless, the usefulness of RNA aptamers has been limited by difficulties in delivering the RNA to target cells and tissues, and only a few examples demonstrate *in vivo* efficacy. Shi *et al.* produced a functional pentavalent aptamer expressed by RNA polymerase II in *Drosophila* [29]. Good *et al.* created a strategy, similar to our strategy, in which human $tRNA_{met}$ and U6 snRNA promoters intracellularly express RNA aptamers that inhibited HIV gene expression [30].

The work presented here describes an approach to generate functional expression cassettes for previously identified RNA aptamers in such a way that the expressed aptamers retain their ability to bind and inhibit the original target protein. Perhaps not surprisingly, the simple insertion of a previously selected aptamer sequence into a pol III expression construct was found to eliminate much of the binding activity of the aptamer. It is likely that the folding conformation of these longer RNA molecules disrupted the structure of the E2F-E1 aptamer sequence enough to alter its ability to bind to and inhibit E2F activity [31–33]. This disruption of the binding integrity of the aptamer presents a serious problem, because it is most efficient to carry out the initial selection in the context of a small RNA molecule rather than the entire expression molecule.

The alternative strategy described here provides an approach to convert a previously selected aptamer sequence to a form that can be used in an *in vivo* setting. The logic of our approach has been to insert the previously selected E2F aptamer sequence into the loop region of the highly expressed S35 construct and to vary the context of this insertion to achieve optimal presentation of the aptamer. Although we do not know if this strategy will be generally successful, it did work in the case of this E2F-specific aptamer, and we believe it is possible that it will be an effective strategy in other cases. Of course, actually using this selected expression aptamer for therapeutic purposes will require additional steps, because simple plasmid-based transfection will not be an effective delivery approach for most *in vivo* applications. However, transferring this expression construct to a viral vector system, such as a recombinant adenovirus, should be straightforward and allow for efficient delivery and expression of the aptamer in target tissues.

MATERIALS AND METHODS

Preparation of RNA polymerase II-expressed E2F-E1 constructs. Overlapping oligonucleotides containing the essential E2F-E1 stem-loop (5′-GTCGATGTGATAAGTAGGACGGAGGTGGTCGAT-3′) [7] were synthesized with a 3′ *Kpn*1 restriction site and 5′ *Sca*1 and *Bam*H1 sites. These oligos were annealed and cloned into the *Kpn*1 and *Bam*H1 sites behind the CMV promoter of a modified pcDNA (Invitrogen, San Diego, CA) construct.

Preparation of RNA polymerase III-driven E2F-E1 constructs. The E2F-E1 stem-loop sequence was inserted into the Δ3-2 expression vector at the *Bam*H1 site. The E2F-E1 stem-loop sequence was similarly inserted into the S35 vector [19]. The Δ3-2-E1 and S35-E1 constructs were then subcloned into the *Sal*I and *Bgl*II sites of bluescript (Stratagene, La Jolla, CA). The template for further selection of E2F-E1 within an RNA polymerase III vector was created as follows. The E2F-E1 stem-loop sequence was inserted into the S35 construct by PCR amplification using the following primers: 5′-AAACAGAACAGCAGAGTGGCTGGGATGNNNNNNNNNNGCTAGCATC-GACCACCTCCGTCCTACTTATCACATCGACGCTAGCNNNNNNNNNNC-ATCCCAGCGCAGCGGTACG-3′, and 5′-TAATACGACTCACTATAGGGCA-GAACAGCAGAGTGGCGCCAGCGG-3′. Once inserted, the construct was amplified using the shorter primer, 5′-AAACAGAACAGCAGAGTG-GCTGGGATG-3′. A T7 promoter is included in this construct for *in vitro* expression. Secondary structure of the S4.2 construct was predicted using the MulFold program [20].

Selection of S35-E2F-E1 constructs for binding to E2F1. Using the randomized S35-E1 construct, approximately 10^{12} unique sequences were transcribed to yield 10^{13} RNA molecules using T7 RNA polymerase. DNA was degraded using DNase I. RNA was then separated on a denaturing urea gel, eluted, and equilibrated in buffer containing 20 mM Hepes, pH 7.9, 150 mM KCl, 6 mM $MgCl_2$, and 1 mM dithiothreitol. The binding assay was performed with GST-E2F1 in the presence of 0.15% bovine serum albumin using 30 nM RNA. The initial cycle of selection was performed with 10^{13} molecules of GST-E2F1 bound to glutathione Sepharose beads (Pharmacia Laboratories, Piscataway, NJ). In subsequent rounds of selection the amount of GST-E2F1 was incrementally decreased to a minimum of 10^{12} molecules by the fourth round. Binding was performed at room temperature for 30 minutes, followed by rinses with the buffer (1 rinse in the initial round and up to 4 rinses in the final round). RNA was then eluted from the beads using 100 mM glutathione, 50 mM Tris, pH 9.0. RNA was extracted with phenol and chloroform, then precipitated in the presence of sodium acetate with ethanol. The RNA was reverse transcribed with AMV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany) in the presence of GC Melt (Clontech, Palo Alto, CA) and 5% glycerol. The product was PCR-amplified using GC Melt, then gel purified on an acrylamide gel with TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA). The DNA was eluted from the gel slice in 10 mM Tris, pH 8, and 0.1 mM EDTA and re-transcribed for the next round of selection. To qualitatively monitor the progress of the selection following each round of SELEX, we determined the minimum number of cycles of PCR amplification that were required to generate enough DNA to be detected on an agarose gel stained with ethidium bromide. This number decreased from 16 cycles, following SELEX round 1, to 8 cycles following rounds 4 and 5. Because this plateau was reached after round 4, the products from round 4 were cloned into the PCR2.1 vector and sequenced. One construct, S4.2, was further modified to S4.2m by replacing the G at residue 134 (residue 22 of the E2F-E1 stem-loop

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sequence described above) with A (Fig. 1), thus disrupting the E2F binding ability.

Selection of molecules from the randomized stem pool started with relatively low stringency and escalated to higher stringency in the binding reaction. Binding reaction stringency was modulated by changing the number of E2F molecules available for binding and by increasing the number of washes with buffer following binding. PCR was carried out for a range of rounds to determine the optimal number required for amplification of the product. The optimal number of rounds was defined as the fewest number of rounds of PCR that resulted in maximal product. The number of rounds of PCR also served as a surrogate indicator of the binding affinity. In early rounds of selection, the number of PCR rounds required was relatively high. In later rounds, this number decreased and reached a plateau. This was interpreted as indicating that the affinity of the pool of RNA species for E2F1 had increased, but could not be significantly enhanced by further rounds of selection.

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay was performed by incubating ^{32}P-labeled DNA probe (*Eco*R1-*Hin*dIII fragment from the ATF(–) adenovirus E2 promoter plasmid at a concentration of 1 nM with 20 nM GST-E2F1 and varying concentrations of inhibitor RNA. Incubation was performed for 20 minutes at room temperature in 10 μl of buffer containing 20 mM Hepes, pH 7.9, 40 mM KCl, 6 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1% NP-40, 10% glycerol, 0.15% BSA, and 500 ng of salmon sperm DNA. Complexes were separated on a 4% polyacrylamide gel in TBE for 3 hours at 300 V at 4°C.

Cell culture. Human 293 cells were obtained from ATCC, and were cultured in the presence of Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY) with 10% fetal calf serum (Hyclone, Logan, UT). Cells from confluent cultures were removed from the plate with light trypsinization (trypsin/EDTA; GIBCO BRL) and replated at a 1:3 to 1:10 dilution.

Northern blot analysis. Cells were lysed using trizol, extracted with chloroform, and precipitated with isopropanol 24 hours following transient transfection of inhibitor constructs. RNA was dissolved in a solution containing 69% formamide, 8.9% formaldehyde, 40 mM morpholino-propanesulfonic acid, 10 mM Na acetate-3H_2O, and 1 mM EDTA, pH 7.2. RNA was separated on a gel containing 2% formaldehyde and MOPS buffer, then transferred to a genescreen membrane (NEN Life Science Products, Boston, MA). The membrane was probed with a ^{32}P-labeled anti-E2F-E1 oligonucleotide (5′-CGTCCTACTTATCACATCGAC-3′). RNA in gel bands was quantified by phosphoimagery and compared to known standards.

Measurement of E2F activity. Promoter transactivation by E2F was evaluated by measuring CAT activity following cotransfection of 4X-E2-CAT [34,35]. The reporter plasmid (CMV β-gal), Bluescript, CMV-E2F1 (0.1 μg), and CMV-DP1 (0.1 μg) were cotransfected by calcium phosphate coprecipitation into 293 cells. Calcium phosphate precipitation was performed by co-precipitating DNA constructs in a buffer solution which, after mixing, contained 125 mM $CaCl_2$, 140 mM NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4-2H_20, 6 mM dextrose, and 25 mM HEPES, pH 7.05. Precipitate was incubated with 293 cells for 3–6 hours followed by 10% glycerol shock for 90 seconds. Cells were scraped from the plates and lysed by freeze-thawing in 250 mM Tris pH 7.8. β-Gal activity was measured with chlorophenol red-β-d-galactopiranoside (CPRG) using a colorimetric assay. CAT activity was measured by incubating cell lysates, normalized to β-gal activity, from approximately 10^5 cells in 130 μl volume containing 625 mM Tris, pH 7.8, 25 nCi [^{14}C]chloramphenicol (56 mCi/mmmol), and 70 μg acetyl coenzyme A for 2 hours at room temperature. The mixture was extracted with ethyl acetate and separated by thin layer chromatography. The acetylated spots were quantified by autoradiography and densitometry.

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