Dual Level Inhibition of E2F-1 Activity by Adeno-associated Virus Rep78*

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E2F-1, a major cellular transcription factor, plays a pivotal role in regulating the cell cycle. The activity of E2F-1 is negatively regulated by its interaction with retinoblastoma protein (pRB), and disruption of the pRB-E2F-1 complex, a hallmark of cellular transformation by DNA tumor viruses, leads to cell proliferation. Adeno-associated virus-2 (AAV) is known to have oncosuppressive properties against DNA tumor viruses. Here we provide, for the first time, the molecular basis for antioncogenic activity of AAV. Rep78, a major regulatory protein of AAV, interacts at the protein level with E2F-1 and stabilizes the pRB-E2F-1 complex. At the DNA level, Rep78 binds to a putative site on the E2F-1 promoter and down-regulates the adenovirus-induced E2F-1 transcription. This dual level of Rep78 activity leads to decreased cellular levels of free E2F-1, leading to its oncosuppressive properties.

Adeno-associated virus-2 (AAV) is a 4.7-kilobase pair, non-pathogenic, single-stranded DNA virus. It requires cellular conditions provided by helper viruses such as adenoavirus, herpesvirus, or vaccinia virus for its productive replication (1, 2). AAV latently infects the cell and integrates preferentially into human chromosome 19 q13.3-qter (3). AAV has been shown to inhibit adenovirus-generated tumors in chicks and hamsters. In vitro experiments have shown that the co-infection of AAV with adenovirus leads to a reduction in the number of plaques generated when compared with infection by adenovirus alone (4, 5). The transforming properties of not only adenovirus (Ad) (4, 6, 7) but also other DNA tumor viruses such as simian virus 40 (SV40) (8–12) and human papilloma virus (13, 14) are inhibited by AAV. These tumor-suppressive and antiproliferative properties have been mapped to the left half of the AAV genome, which codes for the multifunctional regulatory protein Rep78 (15, 16).

Small DNA viruses such as adenovirus, papillomavirus, and SV-40 rely on the host cell for many of the steps needed for their own propagation. They encode proteins that inactivate the function of a key cellular growth-regulatory protein, the retinoblastoma gene product (pRB), to facilitate a productive viral infection in an otherwise quiescent cell (17).

The retinoblastoma protein, pRB, is an important regulator of the G1-S transition (18, 19). pRB negatively influences cell cycle progression by binding to E2F-1, a transcription factor required for expression of genes that are important in cell cycle regulation. It controls the expression of several genes activated in the G1 phase of the cell cycle, which includes its own expression (20, 21), and genes such as cyclin E and p107 (22). E2F-1 also contributes to the cell cycle-regulated expression of a number of genes that are required during S phase, such as dihydrofolate reductase, DNA polymerase a, cyclin A, c-myc, N-myc, and c-myb (23–27). Adenovirus E1A, SV40 large T antigen, and the human papillomavirus E7 proteins disrupt the pRB-E2F-1 complex, releasing unbound or free E2F-1 (28), indicating that E2F-1 is a common cellular target of DNA tumor viruses (29, 30) disrupting the normal cell growth controls.

Because AAV inhibits the oncogenic potentials of such a wide variety of DNA tumor viruses, we investigated whether AAV interferes with the disruption of the pRB-E2F-1 complex, which is the hallmark of all DNA tumor virus-mediated cell proliferation. This report shows that AAV Rep78 acts on E2F-1 at transcription as well as pRB interaction levels to decrease E2F-1 activity and provides a definite molecular mechanism for the antioncogenic property of AAV Rep78.

**EXPERIMENTAL PROCEDURES**

Plasmids—pCMV-E2F-1 was kindly provided by Dr. K. Helin (European Institute of Oncology, Milan, Italy); pE2F-1 Luc (−728) was supplied by Dr. Joseph R. Nevins (Duke University Medical Center, Durham, NC); and GST-E2F-1 and pE2-luciferase were from Dr. Ed Harlow (Harvard University, Boston, MA). pCMV-Rep78 was constructed as follows. AAV Rep78 was PCR-amplified using the Expand high fidelity PCR system (Roche Molecular Biochemicals) with BglII overhangs using linearized plasmid, pAS203. The primers used were 1) Rep321BglF (forward) (5′-CTG CAG ATC TAT GCC GGG GTT TTA CGA G-3′) and 2) Rep2186, BglR (reverse) (5′-CTG CAG ATC TAT GTA TTT GGA AAT GAC GCA G 3′). PCR product was gel-purified (QIAquick PCR Purification Kit; Qiagen Inc., Valencia, CA) and cloned into pBAD/TOPO® ThioFusion vector (Invitrogen Corp., Carlsbad, CA). The resulting plasmid was digested with BglII, and the 1.87-kilobase pair AAV Rep78 was gel-purified. This Rep78 fragment was cloned into a pCMV vector, downstream of the CMV promoter, which was digested with BamHI.

Viruses and Cell Lines—Cell lines (293, HeLa, and normal human fibroblasts) were obtained from ATCC (American Type Culture Collection, Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 1% penicillin G/streptomycin, 2 mm l-glutamine (Life Technologies, Inc.) at 37 °C under a humidified atmosphere containing 5% CO2. AAV type-2 was prepared from HeLa cells infected with adenovirus-2, and AAV-2 and purified as described previously (31). All experiments were carried out with 10 multiplicity of infection units of adenovirus-2 and/or AAV-2 on
semiconfluent fibroblasts HeLa or 293 cells, and the cells were harvested at the described time intervals.

Lipofection and Luciferase Assay—Plasmids pCMV-E2F-1, pCMV-Rep78, pE2F-1 luciferase, and pE2 luciferase were transfected into human cells using the Effectene transfection kit (Qiagen Inc., Valencia, CA), as described by the manufacturer. One day prior to transfection, 10^5 cells were plated per well of a six-well tissue culture plate. DNA (0.5 μg/transfection) was sequentially mixed with enhancer and effectene reagent and layered on monolayer cells. After 2–4 h of incubation, the medium was replaced with fresh regular growth medium, and cells were further subjected to viral infections with adenoviral and/or AAV as described earlier. The cells were lysed inside the wells by using 200 μl of lysis buffer and harvested at described intervals. The luciferase assay was performed using a luciferase assay detection system (Promega Corp., Madison, WI).

RNase Protection Assay—RNase protection assay was performed using the “Riboquant” multiprobe RNase protection assay system (Pharmingen, San Diego, CA). A human cell cycle regulator multiprobe template set, hTS-1 (containing E2F1 and glyceraldehyde-3-phosphate dehydrogenase cDNA sequences) was utilized to synthesize [32P]UTP-labeled antisense RNA probe. Template DNA molecules were digested with RNase-free DNase, and the probe was purified by phenol/chloroform extractions and ethanol precipitation. Purified probe (10^5 cpm) was mixed with 10 μg of total RNA from 293 cells in hybridization buffer. Hybridization was carried out for 16 h at 56 °C. Free probe and single-stranded RNA molecules were digested with a mixture of RNase A and T1. The “RNase-protected” molecules were purified and resolved on a denaturing polyacrylamide gel, dried, and autoradiographed.

Histidine-tagged Rep78 and GST-E2F-1 Protein Purification—Histidine-tagged Rep78 protein was produced and purified as previously described (31). GST-E2F-1 fusion proteins expressed in Escherichia coli were affinity-purified with the MicroSpin™ GST purification module (Amersham Pharmacia Biotech). Purification was carried out under native conditions, essentially following the protocol provided by the company. Briefly, small overnight cultures were transferred to 1 liter of LB broth and cultured until the absorbency (A600) reached 0.7. Then isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM, and the incubation was continued for 4 h after the induction. The cell pellet was suspended in 40 ml of PBS and freeze-thawed three times and incubated with lysozyme at room temperature for 10 min. Later, the extract was centrifuged at 10,000 rpm for 30 min at 40 °C, and supernatant was used for the purification of GST-E2F-1. The purity of both proteins was confirmed by 8% SDS-polyacrylamide gel electrophoresis.

Affinity Chromatography with Rep78—His-Rep78 protein was expressed as described earlier, and protein was adsorbed to Ni²⁺-nitrilotriacetic acid spin columns (Qiagen, Santa Clarita, CA) according to the instructions given by the manufacturer. p53 protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was chromatographed on the Rep78 affinity column by incubating at 4 °C for 30 min. Next, the His-Rep78 was eluted with 250 mM imidazole and subjected to 8% SDS-polyacrylamide gel electrophoresis. Some blots were also transferred to nitrocellulose membrane as mentioned earlier for probing with antibodies.

Affinity Chromatography with GST-E2F-1—Approximately 100 ng of GST-E2F-1 already bound to the MicroSpin™ GST purification module (Amersham Pharmacia Biotech) was preincubated for 1 h at room temperature in 0.5 ml of 0.25% gelatin, 50 mM KCl, 50 mM HEPES (pH 7.4). Dual Level Inhibition of E2F-1 Activity by Rep78

FIG. 1. Alterations in the E2F-1 levels with AAV infection. A, viral infections were carried out on 60–80% confluent HeLa cells as mentioned under “Experimental Procedures.” Cell extracts were prepared at the indicated intervals and, following Western blotting, were probed with E2F-1 polyclonal antibodies and probed with anti-Rep antibodies. Proteins were visualized by enhanced chemiluminescence. B, human diploid fibroblasts and 293 cells were infected with viruses, and the cell extracts were prepared and probed as above with E2F-1 polyclonal antibody. The time of optimal Rep78 expression is shown here. C, RNase protection assay with E2F-1 probe on RNA isolated from human diploid fibroblasts 96 h after viral infection. Numbers below E2F-1 represent relative densitometric readings of signal intensity.
**Fig. 2. Binding of AAV Rep78 to the putative binding site on E2F-1 promoter.**

A. The nucleotide sequence of E2F-1 promoter with highlighted and underlined minimal tetrameric AAV Rep78 binding motif, GCTC. The E2F-1 binding motif, TTTCGCG, on its own promoter immediately adjacent to the Rep78 binding motif is highlighted. B. Gel mobility shift analysis shows a dosage-dependent increase in the specific interaction of AAV Rep78 with its homologous binding region on the E2F-1 promoter (E2F1p) (lanes 1–3), competitive inhibition with a cold competitor, the known AAV Rep78 binding sequence (AAV ITR) (lanes 4 and 5), and a lack of inhibition with the scrambled homologous binding region of AAV Rep78 on the E2F-1 promoter (E2F1p) (lane 6). C, the gel shift of AAV ITR with purified Rep78 is successfully competed with the AAV Rep78 binding region on the E2F-1 promoter (E2F1p) as a cold competitor, while its scrambled sequence (E2F1p–) is unable to affect the band shift. E2F1p–, oligonucleotide sequence of putative binding site on E2F-1 promoter. E2F1p, scrambled oligonucleotide of Rep78 binding site on the E2F-1 promoter.

**Electrophoretic Western Blotting—** Aliquots of total protein extracts (100 μg) from cells after different virus treatments were suspended in Laemmli sample buffer (0.1 M Tris-Cl buffer, pH 6.8, containing 1% SDS, 0.05% β-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue) and boiled for 2 min and applied on either 8, 12, or 10–12% (32) glycerol gradient SDS-acrylamide along with a 10-kDa bromphenol blue marker. Samples were normalized to 10 μg/ml with lysis buffer, aliquoted, and estimated using the Micro BCA kit (Pierce). Protein contents of all samples were normalized to 10 μg/ml with lysis buffer, aliquoted, and stored at −70 °C. Immunoprecipitations were conducted as described previously (31).

**Electrophoretic Mobility Shift Assay—** Oligomers used in the assay were custom synthesized (Integrated DNA Technologies Inc., Coralville, IA) and 32P-labeled with T4 polynucleotide kinase. AAV inverted terminal repeats (ITR) used in the assay were generated using three separate oligonucleotide ligations: AAV TR1, 5′-TGA GCC CGG CCG GCC AAA GCC CGG CGG TCG GCC GAC CTT TGG TCC GCC GGC CTC ATG GAG C-3′; AAV TR2, 5′-CAC TCG CTC GCT CGG CGC TCT CCT CAC CGG TTT AAG TAG TGA TCC CCA ATG GA-3′; and AAV TR3, 5′-AGG CGA CGG CGG AGA GGA GTG GCC GGC AAC ACT CAT CAC TAC GGG TCT CT-3′. The E2F-1 promoter containing three Rep78 binding consensus sequence tetramers, GCTC together with the adjacent E2F-1 binding site, were synthesized, and complementary sequences were annealed before radiolabeling. This double-stranded oligonucleotide is referred to as E2F1p. A scrambled sequence of the three Rep78 binding tetrameric sequences of both polarity was custom synthesized and annealed, and this double-stranded oligonucleotide is referred to as E2F1p–. Mobility shift assays were essentially conducted as described earlier (10).

**RESULTS**

**AAV-mediated Inhibition of E2F-1 Gene Expression—** An adenovirus-mediated increase in E2F-1 trans-activation activity is essential for activation of various genes leading to DNA amplification and cell proliferation (33). Because AAV inhibits the adenovirus and other DNA virus-mediated cellular DNA replication and cell proliferation, we examined the expression of E2F-1 protein in the presence or absence of AAV in adenovirus-infected human cells. Cell extracts were prepared from

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**Biochemistry**

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HeLa, infected at various time points with adenovirus and/or AAV, and analyzed by Western blot with E2F-1 antibodies. Adenoviral infection dramatically increased the E2F-1 protein levels that appeared at 24 h and reached maximum at 48 h after viral infection. The increased E2F-1 levels were significantly reduced upon co-infection with AAV (Fig. 1A). To correlate the changes in E2F-1 levels with AAV Rep protein expression, we probed the cell lysates with anti-Rep antibody. Significant Rep protein expression was observed at 48 h after the infection and reached a plateau by 72 h, correlating with the maximum inhibition in the E2F-1 levels (Fig. 1A). To eliminate any bias associated with a particular cell line, we have evaluated these changes in two additional cell types: normal human fibroblasts and 293, a human kidney cancer cell line. Similar changes in E2F-1 levels were observed in normal human fibroblasts (Fig. 1B, lanes 1–4) and in 293 cells (Fig. 1B, lanes 4–8), coinciding with maximal AAV Rep protein expression in these cell types. A RNase protection assay performed three times in normal human fibroblasts revealed an overall 7.5-fold decrease in the E2F-1 RNA level upon AAV infection of adenovirus-infected fibroblasts (Fig. 1C). These results indicate that adenoviral mediated up-regulation of E2F-1 expression is significantly inhibited upon AAV infection, which is reflected at both RNA and protein levels.

AAV Rep78 Binds to E2F-1 Promoter—Rep78 is known to bind to various heterologous promoters, leading to inhibition of the transcription (15, 34, 35). Previous experiments indicated that AAV inhibits adenovirus-mediated up-regulation of E2F-1. To understand the mechanism behind AAV-mediated inhibition of E2F-1 gene expression, we evaluated the E2F-1 promoter sequence that revealed Rep78 DNA-binding motifs, GCTC (8, 36, 37), proximal to the binding sites for E2F-1 on its own promoter element (Fig. 2A). Homologous binding sites for Rep78 on the E2F-1 promoter raised the possibility of its interaction and possible control of transcription of the E2F-1 gene. We designed a 35-base pair oligonucleotide of the E2F-1 promoter sequence encompassing the putative Rep78 and E2F-1 binding sites (E2FR1) and a mutated version where the Rep78 binding sequence is scrambled (E2FR2). A gel shift assay was used to determine whether Rep78 binds to these homologous motifs. We observed a dose-dependent increase in the binding activity of purified Rep78 protein to the homologous binding sites on E2F-1 promoter (Fig. 2B, lanes 1–3). The band shifts were successfully competed by AAV ITR, a known DNA binding sequence of Rep78, indicating the specificity of binding (Fig. 2B, lanes 3–5). The specificity of binding was further confirmed by lack of competition by cold mutated E2F-1 oligonucleotide with a scrambled Rep78 binding sequence (E2FR2), (Fig. 2B, lane 6).

To further confirm the specificity of Rep78 binding to the E2F-1 promoter sequence, a change in band shift of known Rep78 binding DNA sequences (AAV ITR) with affinity-purified Rep78 was evaluated with E2F-1 promoter sequences. Cold oligonucleotide containing the Rep78 homologous DNA binding motif, GCTC, was used to confirm the specificity of Rep78 binding to the E2F-1 promoter sequence (Fig. 2B, lane 5). These results indicate that Rep78 binds to the E2F-1 promoter sequence, leading to inhibition of transcription.
motif on the E2F-1 promoter (E2F1_2) was able to compete with the gel shift, while mutated homologous Rep78 binding motif on E2F-1 promoter (E2F1_3) was unable to compete with the band shifts generated by AAV ITR and Rep78 protein (Fig. 2C). This strongly suggests the specific binding of AAV Rep78 to its putative binding motif on the E2F-1 promoter.

**Functional Inhibition of E2F-1 Promoter Activity by AAV**—To investigate the functional implications of AAV Rep78 binding to the E2F-1 promoter, we evaluated E2F-1 promoter activity in the presence or absence of AAV in adenovirus-infected cells. Cells were transfected with a E2F-1-luciferase plasmid construct (pE2F1-Luc) followed by adenovirus and/or AAV infections, and cell extracts were prepared as noted under “Experimental Procedures.” Luciferase activity measured in these extracts directly reflects the E2F-1 promoter activity. The adeno-virus-mediated increase in the E2F-1 promoter activity was drastically reversed to basal levels upon AAV co-infection in normal human fibroblasts (Fig. 3A), 293 cell line (Fig. 3B), and HeLa cell line (Fig. 3C) at time points that coincided with the AAV gene expression in these cells (p < 0.001). Interestingly, AAV alone also inhibited the E2F-1 activity, although the conditions in the absence of helper virus do not allow maximal expression of Rep78. To specifically evaluate the role of Rep78 in the observed AAV-mediated inhibition of E2F-1 transcription, we conducted the same set of experiments using transfection of a eukaryotic expression plasmid containing the full-length Rep78 gene under CMV promoter control (pCMV-Rep78) instead of AAV infection. The adenovirus-mediated increase in the E2F-1 transcriptional activity, as determined by luciferase assay, was dramatically reversed to basal levels with the co-transfection of pCMV-Rep78 plasmid in all three cell types (Fig. 3, D–F) (p < 0.001). Combined, these experiments indicate that AAV through Rep78, interferes with the E2F-1 gene expression to inhibit the adenovirus-mediated up-regulation.

**AAV Stabilizes the pRB-E2F-1 Complex from Adenoviral E1A-mediated Dissociation**—Apart from the control at transcriptional level, activity of E2F-1 is also controlled through its binding to pRB. However, binding of adenoviral E1A to the pRB in adenovirus-infected cells results in the dissociation of pRB-E2F-1 complex (38). To investigate the effect of AAV on E1A-induced dissociation of the pRB-E2F-1 complex, cellular extracts with and without AAV/Ad treatments were immunoprecipitated with anti-pRB antibodies, and the complex was resolved on 8–12% gradient SDS-polyacrylamide gels, transferred onto nitrocellulose, and probed with E2F-1 antibodies in fibroblasts, 293 cells, and HeLa cells. In both untreated cells and cells treated with AAV alone (normal human fibroblasts, Fig. 4A, lanes 1 and 2; 293 cells, Fig. 4B, lanes 1 and 2; and HeLa cells, Fig. 4B, lanes 5 and 6), E2F-1 co-immunoprecipitated with pRB antibodies. As expected, E2F-1 did not co-immunoprecipitate with pRB in adenovirus-infected cells, indicating that the pRB-E2F-1 complex is dissociated in these cell types (Fig. 4, A, lane 3, and B, lanes 3 and 7). However, co-infection of adenovirus-infected cells with AAV showed E2F-1 co-immunoprecipitation with pRB antibodies, confirming the presence of Rb-E2F-1 complex in all three cell lines (Fig. 4, A, lane 4, and B, lanes 4 and 8). Additionally, we have analyzed the immunodepleted lysate from normal human fibroblast showing that, following pRB precipitation of adenovirus-infected cell lysate, there is a high level of E2F-1 remaining in the immunodepleted lysate; however, as the complex is stabilized in the presence of AAV Rep78, the amount of free E2F-1 is decreased in the immunodepleted lysate (Fig. 4A, lanes 5 and 6), further indicating that pRB and E2F-1 are bound in adenovirus+AAV samples.

**Interaction of AAV Rep78 with E2F-1 and pRB**—Since we did not observe any change in the adenoviral E1A levels or its binding to pRB following AAV superinfection of adenovirus-infected cells (data not shown), we hypothesized that AAV Rep78 may directly bind to either pRB or E2F-1, resulting in the inhibition of the E1A-mediated dissociation of the complex. Cell lysates were immunoprecipitated separately with E2F-1 and pRB antibodies and probed for the presence of Rep78 protein. We observed AAV Rep78 and Rep52 (spliced product of Rep78) in the immunoprecipitates with both pRB and E2F-1 antibodies, indicating interaction of AAV Rep78 with both E2F-1 or pRB or both, leading to the stabilization of the complex (Fig. 5A). To further analyze whether Rep78 interacts with pRB or E2F-1, we investigated in vitro interaction between purified Rep78 with purified pRB and E2F-1 proteins. AAV Rep78 affinity columns were made, and different amounts of E2F-1 were passed through the columns. Bound proteins were eluted with imidazole, and the resulting extracts were subjected to Western blot analysis using Rep78 and E2F-1 antibodies. We observed interaction of E2F-1 protein with AAV Rep78 as seen by the co-elution of Rep78 with E2F-1 (Fig. 5B, lane 1). To exclude the possibility that GST E2F-1 protein may interact with a histidine tag on Rep78 protein, we passed the GST-E2F-1 alone in the histidine affinity column in the absence of AAV Rep78. As seen in Fig. 5B, lane 2, GST-E2F-1
does not bind to the histidine affinity column. We have also carried out GST-E2F-1 affinity chromatography, where the GST-E2F-1 affinity columns were prepared as mentioned in the methods and incubated with increasing concentrations of purified AAV Rep78. Bound GST-E2F-1 was eluted with reduced glutathione, and the resulting extracts were probed for AAV Rep78 by Western blot analysis. A dose-dependent increase in the binding of AAV Rep78 is observed with GST-E2F-1 (Fig. 5C, lanes 2–4). AAV Rep78 alone does not show any binding to the GST affinity column in the absence of GST-E2F-1 (lane 3). Rep78 column eluted without E2F-1 incubation. C. Rep78 binding to E2F-1 in vitro. E2F-1 was produced as a GST fusion protein, and the affinity matrix was made with the MicroSpin™ GST purification module. Protein incubations and elutions were carried as mentioned under “Experimental Procedures,” and the eluents were subjected to Western blotting with Rep monoclonal antibodies. Lane 1, 50 ng of purified His-Rep78 chromatographed in the MicroSpin™ GST purification module without GST-E2F-1. Lanes 2–4, increasing amounts of purified Rep78 (10, 20, and 60 ng) passed through GST-E2F-1 affinity column. D. Lack of interaction between Rep78 and pRB. Purified His-Rep78 was conjugated to Ni⁺⁺-nitrilotriacetic acid columns, and purified pRB was incubated with resin-bound Rep78 and eluted with imidazole. Eluents were subjected to Western blotting with both Rep and pRB monoclonal antibodies. Lane 1, pRB incubated in Rep78 column. Lane 2, pRB protein incubated in histidine affinity column without Rep78. Note the absence of the pRB band near 110 kDa. Lane 3, GST-E2F-1 and pRB complexed in vitro were added to the His-Rep column, and the eluents were probed with pRB and E2F-1 monoclonal antibodies.

**DISCUSSION**

Progression of cells from one phase of the cell cycle to the next is regulated by transformation-sensitive checkpoint genes (39). The pRB regulates progression of the cell past the restriction point in G1 by negatively regulating the activity of E2F-1 (18, 19, 40). The normal activity of E2F-1 is regulated at two different levels. First, the abundance of E2F-1 is regulated at
FIG. 6. Rep78 inhibits E2F-1-responsive E2 promoter activity. Approximately 0.5 μg of pE2 luciferase plasmids was transfected into normal human fibroblasts (A), 293 cell line (B), and HeLa cell line (C) by lipofection. pCMV-Rep78 transfections were carried out as indicated. After overnight incubation, cells were further subjected to adenoviral infection. Cell lysates were prepared, and luciferase activity was measured in light units. Data presented are means of three experiments and are statistically significant in regard to decrease in Rep78 + Ad compared with Ad alone (p < 0.001).

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the level of transcription. For example, E2F-1 is undetectable in quiescent cells but is transcriptionally induced following mitogenic stimulation in late G1. (20) Second, transactivation by E2F-1 is negatively regulated by complex formation with pRB (41).

The small DNA tumor viruses such as adenovirus, SV40, and the human papillomavirus have evolved specific early genes to disrupt the pRB and E2F-1 interactions, thereby effectively promoting cell proliferation (17, 42) and also indicating that E2F-1 is a common cellular target of DNA tumor viruses (29, 30). Adenovirus requires E2F-1 transcription factor for two purposes: to interact with adenovirus E4 protein and transactivate the E2 promoter (28) and to activate transcription of multiple growth-responsive cellular genes that contribute to cellular and viral DNA synthesis (22, 27). The primary event in adenovirus-mediated cellular transformation is to enhance the cellular “free E2F-1” levels (28, 43). It is in this context that we evaluated E2F-1 as a primary target for AAV in mediating anti-transforming effects. Co-infection with AAV clearly reduces the E2F-1 protein levels in adenovirus-infected cells, and the effect is mediated at the transcription level. Additionally, the level of free E2F-1 is further reduced by inhibiting dissociation of the pRB-E2F-1 complex. The ability of a single protein (Rep78) to inhibit function of a single target gene at both the transcriptional level and at the protein-protein interaction level is interesting. Although the function of Rep78 is similar to pRB in that it binds to E2F-1 and keeps it functionally dormant, there are no sequence similarities between pRB and Rep78. Although the mechanism behind the trans-inhibitory activity of Rep78 is not yet understood, the position of Rep78 binding site on E2F-1 promoter that lies within one base pair of the E2F-1 binding site raises an interesting possibility of Rep78 interfering with autoactivation by E2F-1 of its own promoter. Further studies are required to identify such a relationship.

The activity of Rep78 on an important cellular target, E2F-1, also explains the ability of Rep78 to inhibit the transforming activity of a variety of DNA tumor viruses and also its antiproliferative activity in normal untransformed cells. The diminished levels of E2F-1 with AAV infection in primary human cells in the absence of adenoviral infection were recently reported (44), which further supports the activity of AAV Rep78 on E2F-1, even in the absence of transforming virus infection. Since E2F-1 is known to have autoregulatory activity, it is also possible that the observed down-regulation of the E2F-1 promoter is resultant of the activities of both Rep78 and E2F-1. However, autoregulation of E2F-1 promoter activity has been reported to be only modest (3.5-fold) (20). In our observation in three different cell lines, down-regulation of adenovirus-induced E2F-1 activity by either AAV (Fig. 3, A–C) or Rep78 was between 6- and 10-fold, indicating that the binding of Rep78 to E2F-1 promoter does contribute to the down-regulation of the transcriptional activity. Evolution of AAV Rep78 remains an enigma; however, inclusion of two important checkpoints of E2F-1 function in one protein product raises the possibility that these functions may have later separated into various protein products capable of controlling E2F-1 transcription and its interaction and dissociation from pRB.

The functional consequence of reduced free E2F-1 is confirmed using an E2F-1-responsive promoter with multiple E2F-1 binding sites. That similar effects are reproduced using transfection of CMV-Rep78 plasmid as with wild-type AAV provides compelling evidence that Rep78 is responsible for AAV’s functional anti-transforming activity.

The model of how AAV inhibits the oncogenicity of DNA viruses and possibly other transformation events is becoming more clear. Because the primary targets for the DNA tumor viruses are tumor suppressor genes, p53 and pRB, AAV Rep78
may target this level of cellular control of proliferation. We recently observed that AAV in fact protects the adenovirus-mediated destruction of p53 (31). In this report we show that it affects E2F-1 function not only by preventing Rb-E2F-1 disassociation but also at transcription level, so that overproduction of E2F-1 does not overcome the ability of pRB to regulate E2F-1 function (45). Together, these experiments provide the evidence that Rep78 may be able to reverse some of the major early molecular events necessary for cellular transformation.

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