

The Use of In Vitro Transcription to Probe Regulatory Functions of Viral Protein Domains

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Summary

Adenoviruses (Ads), like other DNA tumor viruses, have evolved specific regulatory genes that facilitate virus replication by controlling the transcription of other viral genes as well as that of key cellular genes. In this regard, the E1A transcription unit contains multiple protein domains that can transcriptionally activate or repress cellular genes involved in the regulation of cell proliferation and cell differentiation. Studies using in vitro transcription have provided a basis for a molecular understanding of the interaction of viral regulatory proteins with the transcriptional machinery of the cell and continue to inform our understanding of transcription regulation. This chapter provides examples of the use of in vitro transcription to analyze transcriptional activation and transcriptional repression by purified, recombinant Ad E1A protein domains and single amino acid substitution mutants as well as the use of protein-affinity chromatography to identify host cell transcription factors involved in viral transcriptional regulation. A detailed description is provided of the methodology to prepare nuclear transcription extract, to prepare biologically active protein domains, to prepare affinity depleted transcription extracts, and to analyze transcription by primer extension and by run-off assay using naked DNA templates.

Key Words: In vitro transcription; nuclear transcription extract; protein affinity; chromatography; run-off transcription assay; primer extension assay; purification of E1A protein mutants; adenovirus protein domains; gene regulation; transcription activation; transcription repression; early gene 1A.

1. Introduction

Adenoviruses (Ads), like other DNA tumor viruses, have evolved specific regulatory genes that facilitate virus replication by controlling the transcription of other viral genes as well as that of key cellular genes that regulate cell cycle progression and cellular DNA synthesis. In this regard, the early gene 1

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(E1A) transcription unit, which encodes two major proteins of 243 and 289 amino acids, is of particular interest. The 289R protein is identical to the 243R protein except that it contains in addition conserved region 3 (CR3, residues 140–188), a powerful transcriptional activator of Ad early genes (*see* **ref. 1** for review). The 243R protein encodes diverse biological functions, including the ability to induce cell cycle progression, immortalize cells, transform cells in cooperation with other oncogenes, and paradoxically to inhibit tumorigenicity and cell differentiation (**1**). These E1A 243R functions are encoded within multiple protein domains that can transcriptionally activate or repress cellular genes involved in the regulation of cell proliferation and cell differentiation. Conserved region (CR)1 (amino acids 40–80) and CR2 (amino acids 120–139) are common to both 243R and 289R and together with the common nonconserved N terminus (amino acids 1 to 39) are required for the growth-regulatory functions of E1A. Exon 2 possesses an autonomous transformation suppression activity within CR4 localized within a 14-amino-acid region near the common C terminus of E1A (**2**). An interesting function that maps to within the E1A N-terminal 80 amino acids is the ability to repress transcription from genes involved in cell proliferation and cell differentiation. To investigate the mechanisms of E1A transcriptional regulation, our laboratory developed an in vitro transcription assay that accurately measures transcriptional activation and transcriptional repression by E1A protein domains (**3**).

Protein–protein binding studies have shown that viral regulatory proteins such as E1A can interact with sequence-specific transcriptional activators as well as with several general transcription factors (GTFs) of the cellular transcription machinery. The significance of these protein–protein interactions in transcriptional regulation can be addressed mechanistically by the application of in vitro transcription methodology using purified recombinant viral proteins.

Transcription in mammalian cells by RNA polymerase II is a complex process that involves the formation of preinitiation complexes composed of at least 44 distinct polypeptides that can be classified into several groups (*see* **ref. 4** for review). A first group of polypeptides constitutes the general transcription machinery and includes RNA polymerase II and the GTFs TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, and TFIIF. A second group consists of nucleic acid sequence-specific transcriptional activators that stimulate transcription, at least in part, by increasing the number of functional transcription complexes. A third group consists of a continually growing number of proteins classified by function as coactivators, positive cofactors, negative cofactors, and general repressors of transcription. This third group activates or represses transcription through protein–protein interactions with transcription factors, often components of the general transcription machinery. The E1A proteins do not bind to

specific DNA sequences, and their transcriptional activities best fit into the third group of transcription factors that function through protein–protein interaction.

The molecular mechanisms of transcriptional regulation by viral protein domains can be effectively explored using *in vitro* transcription systems. Of particular importance was the development of a procedure to prepare extracts from nuclei of HeLa cells that can direct accurate transcription initiation *in vitro* from RNA polymerase II promoters, as described by Dignam et al. (5). Transcriptionally active nuclear extracts can be prepared from different cell types and from as few as 3×10^7 cells (6). The preparation of transcription extracts and the assay conditions can be modified to optimize transcription of specific genes (*see Note 1*). Of additional value is the use of protein-affinity and antibody-depletion experiments to define components within nuclear extracts that interact with specific viral or cellular proteins and are involved in transcriptional regulation. Finally, given the cloning and expression of the transcription factors that comprise the general transcription machinery, it is possible to analyze the functions of viral proteins in a defined reconstituted transcription system. Such studies have provided a basis for a molecular understanding of the interaction of viral regulatory proteins with the transcriptional machinery of the cells and continue to inform our understanding of transcription regulation.

More recent developments relevant for the analysis of viral transcriptional regulators is the recognition that chromatin remodeling plays an important role in transcription regulation. Transcription *in vivo* is regulated at multiple levels from chromatin templates (7). The packaging of DNA into chromatin affects its accessibility by the transcription machinery. Specific patterns of modifications at the histone tails serve as markers for the recruitment of different protein complexes that regulate chromatin structure and gene expression (8). Promoters in which the chromatin–histone structure is unaltered are completely repressed transcriptionally. Interaction with chromatin-modifying activities, such as methylases, acetylases, and phosphorylases, allows for exposure of the promoter so that basal transcription can occur. This basal activity may be in turn modulated by the large number of gene-specific as well as global transcription factors that may activate or repress basal transcription, i.e., transcriptional activators and transcriptional repressors, discussed above. The usual analysis of *in vitro* transcription using “naked” DNA templates most likely reflects the transcription of promoters that are in a “basal state.” The ability of E1A to interact with several histone acetylases and components of the Swi/Snf complex suggests that E1A may regulate transcription at the chromatin level as well as at the basal level. Chromatin assembly *in vitro* has been described (7,9). Development of *in vitro* transcription systems that utilize chromatin templates

in addition to naked DNA allows for another level for understanding transcriptional regulation in a setting that may more accurately reflect that physiological state of cellular gene promoters. In vitro transcription using chromatin templates has been developed to recapitulate transcriptional regulation, which occurs on chromatin in vivo. In vitro transcription studies using chromatin templates will facilitate the understanding of the regulation of gene expression by viral regulatory proteins.

This chapter provides brief examples of the use of in vitro transcription to analyze transcriptional activation and transcriptional repression by purified, recombinant E1A protein domains and single amino acid substitution mutants, as well as the use of protein-affinity chromatography to identify host cell transcription factors involved in viral transcriptional regulation. Following these is a detailed description of the methodology to prepare the nuclear transcription extract, to prepare biologically active protein domains, to prepare affinity-depleted transcription extracts, and to analyze transcription by primer extension and by run-off assay using naked DNA templates.

1.1. Using In Vitro Transcription to Demonstrate the Autonomous Transactivation Activity of Ad E1A Conserved Domain 3

Analysis of E1A mutants by transient expression has demonstrated that CR3 is essential for transactivation of early viral genes by E1A 289R (for review, *see ref. 1*). To determine whether CR3 is sufficient for transactivation, a 49-amino-acid peptide encoding CR3 plus 3 amino acids in exon 2 (termed PD3) was chemically synthesized and tested for its ability to transactivate Ad promoters in vitro. In vitro transcription products were analyzed by primer extension analysis to ensure that transcription initiated at the correct start site. PD3 (100–500 ng), when added to a reaction mixture containing 500 ng of DNA template, either the Ad early region 3 (E3) promoter or the Ad major late promoter, stimulated transcription 5- to 20-fold (*see Fig. 1*). These results show directly that the sequences within CR3 are responsible for E1A transactivation (*10*). Further, these results demonstrate that E1A protein domain CR3 is sufficient for transactivation activity.

1.2. Using In Vitro Transcription to Analyze Transcriptional Repression by the Ad E1A N-Terminal 80-Amino-Acid Sequence

Analysis of E1A mutants by transient expression had indicated that the transcriptional repression function of E1A 243R requires sequences within the nonconserved N-terminal 40 amino acids, within CR1, and also within CR2 in some cases (for review, *see ref. 1*). In order to permit a biochemical definition of the viral and cellular components involved in the E1A repression function, it was important to establish a system that faithfully recapitulates E1A repression in vitro. A recombinant protein containing only the E1A 80 N-terminal

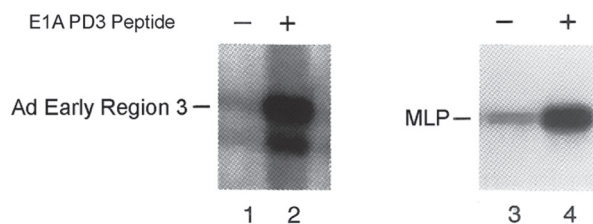


Fig. 1. In vitro transcription of the adenovirus (Ad)2 E3 promoter and the Ad2 major-late promoter (MLP) in the presence (+) and absence (-) of E1A PD3 peptide (CR3 plus three amino acids of Exon2). In vitro transcription was performed with reaction mixtures containing E3 or MLP plasmids as templates and subjected to primer extension analysis. Primers consisted of 5'-end-labeled synthetic oligonucleotides complementary to the E3 mRNA (positions +108 to +137) or MLP mRNA (positions +67 to +86) from the start site of transcription. Labeled cDNA products were analyzed by electrophoresis on a 6% polyacrylamide—7*M* urea gel followed by autoradiography.

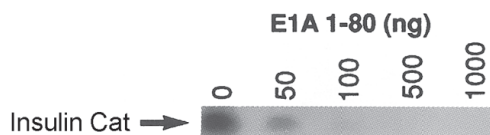


Fig. 2. Transcription repression in vitro of the insulin II promoter by purified recombinant E1A 1-80 protein. pInsulin-CAT was used as template for the in vitro transcription reaction. Transcripts were analyzed by primer extension using a CAT primer followed by autoradiography. Reaction mixtures contained from 0 to 1000 ng of E1A 1-80, as indicated.

amino acids (E1A 1-80), which includes the N terminus plus all of CR1, as well as a series of E1A 1-80 deletion mutant proteins were prepared (*see Sub-heading 3.2.*). These proteins were used to study in vitro transcriptional repression of promoters previously reported by transient expression to be E1A repressible, including those of insulin, interstitial collagenase, simian virus 40, and HIV-long terminal repeat (LTR). Assay conditions by primer extension were first established for in vitro transcription of each promoter fused to the CAT reporter gene (3). The addition of E1A 1-80 protein to the transcription mixture strongly represses these promoters in a dose-dependent manner (*see Fig. 2*). Repression is promoter-specific because promoters not repressed by E1A in vivo are not repressed by E1A in vitro. Further, repression is E1A sequence specific, as shown by the analysis of E1A 1-80 deletion mutant proteins (3). Thus, the in vitro transcription-repression assay faithfully reflects E1A repression and provides a valuable system to study molecular mechanism, as described below.

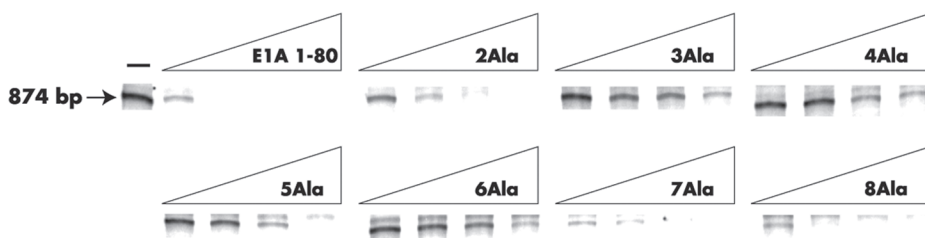


Fig. 3. In vitro transcription repression by single amino acid substitution mutants within the Ad E1A 1-80 polypeptide. Shown are run-off in vitro transcription reactions measuring the transcription repression abilities of E1A 1-80 polypeptide and the first seven E1A 1-80 mutants with single substitutions of alanine for the indicated residue. Reaction mixtures contained between 62.5 and 500 ng of polypeptide. Substitution of 3His, 4Ile, 5Ile, or 6Cys with Ala severely interfered with the ability of E1A 1-80 to repress transcription from the HIV long tandem repeat promoter.

1.3. Using In Vitro Transcription to Identify Amino Acids Critical for the Repression Function Within the E1A 1-80 Polypeptide

Single amino-acid substitution in each of the first 30 amino acids of E1A 1-80 were constructed and the mutant polypeptides were expressed in *Escherichia coli*, purified and renatured as described in **Subheading 3.2**. Each amino acid was substituted in turn with alanine except where alanine was the natural occurring amino acid; in this case the amino acid was substituted with glycine. Alanine scanning mutants have the advantage of removing all amino acid side-chain elements and have been successfully used to identify critical amino acids in several transcriptional regulatory proteins (*see ref. 11* for example). E1A 1-80 and the alanine scanning mutant polypeptides were used in run-off transcription reactions with an HIV LTR promoter as template. **Figure 3** shows that E1A 1-80 efficiently represses transcription over the range of concentrations tested, whereas substitution of amino acids 3, 4, 5, and 6 (and 20 not shown in this figure) with alanine resulted in polypeptides that are deficient in transcription repression activity. Of significance, the amino acid substitution mutants defective for in vitro repression activity are also defective for in vivo repression activity as measured by cell microinjection (*12*).

1.4. Using Protein-Affinity Depletion of Nuclear Extracts to Identify Transcription Factors That Interact With the E1A Repression Domain

The ability of E1A 1-80 to repress transcription in vitro strongly implies that E1A interacts with a cellular protein(s), presumably a transcription factor(s). In order to understand the mechanism of E1A repression, it is important to identify the cellular target(s) of E1A functional domains. E1A 1-80 protein-

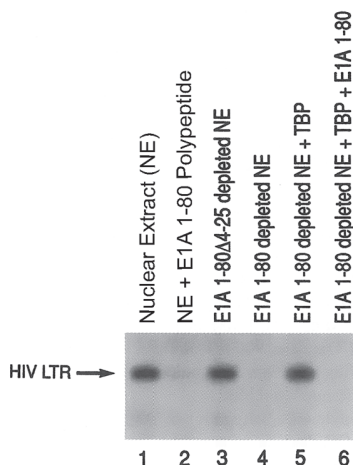


Fig. 4. TATA binding protein (TBP) can restore transcriptional activity to an E1A 1-80-depleted nuclear extract. In vitro transcription and primer extension analysis was performed with pBennCAT (HIV long tandem repeat) as template and a CAT primer. The transcriptional activity of the original nuclear extract (NE) (lane 1) is repressed by added E1A 1-80 protein (400 ng, lane 2). Transcriptional activity is lost by passage through an E1A 1-80 affinity column (lane 4) but not an E1A 1-80Δ4-25 column (lane 3). The addition of TBP (5 ng, lane 5) restores transcriptional activity to the E1A 1-80 affinity depleted NE. The activity restored by TBP can be repressed by addition of E1A 1-80 (400 ng, lane 6).

affinity chromatography was performed to sequester and identify cellular factor(s) that interact with the E1A N-terminal sequence (**13**). A complete loss of transcription activity occurs when a nuclear extract is passed through a column containing immobilized E1A 1-80 protein, but no loss of activity occurs when the extract is passed through a column containing the repression-defective mutant protein, E1A 1-80Δ4-25 (**Fig. 4**). These results provide strong presumptive evidence that the E1A repression domain interacts specifically with and depletes an essential transcription factor(s) from the nuclear extract. Western analysis demonstrated that the GTF, TBP (TFIID), was depleted from the extract and was bound to E1A 1-80 (**13**). Of significance, activity of the depleted extract is completely restored by the addition of TBP, thus providing strong evidence that TBP (TFIID) is a target of E1A repression (**Fig. 4**).

2. Materials

To minimize RNase contamination, all reagents should be made with water that is known to contain less than 20 ppm total organics. Alternately, DEPC-treated water may be used: add 1 mL of DEPC per L of water, shake well, and

autoclave after 1 h. Wear gloves during all operations to avoid contamination with finger RNases. Pipetting devices should be wiped down with ethanol and never used with solutions containing RNase. All reagents can be stored at -20°C .

1. Phosphate-buffered saline (PBS): 1.54 mM KH_2PO_4 , 155.17 mM NaCl, 2.71 mM Na_2HPO_4 .
2. Buffer A: 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) (pH 7.9 at 4°C), 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM dithiothreitol (DTT).
3. Low-salt buffer: 20 mM HEPES (pH 7.9 at 4°C), 1.5 mM MgCl_2 , 20% glycerol, 20 mM KCl, 0.2 mM ethylene diamine tetraacetic acid (EDTA), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM DTT.
4. High-salt buffer: 20 mM HEPES (pH 7.9 at 4°C), 1.5 mM MgCl_2 , 20% glycerol, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT.
5. Buffer D: 20 mM HEPES (pH 7.9 at 4°C), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT.
6. QIAGEN buffer A: 100 mM NaH_2PO_4 , 10 mM Tris-HCl, pH 8.0, 6 M guanidine-HCl (adjusted to pH 8.0 with NaOH).
7. 0.5X buffer D: 10 mM HEPES (pH 7.9 at 4°C), 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 0.1 mM PMSF, 0.25 mM DTT.
8. 20 mM HEPES, pH 7.2.
9. 1 M Ethanolamine, pH 8.0.
10. Forward exchange buffer (10X): 500 mM Tris-HCl (pH 7.5), 100 mM MgCl_2 , 50 mM DTT, 1 mM spermidine.
11. Transcription buffer (10X): 40 mM HEPES (pH 7.9 at 4°C), 40 mM creatine phosphate, 100 mM MgCl_2 , 200 mM KCl, 5 mM DTT, 0.2 mM EDTA.
12. Stop mix: 20 mM EDTA, pH 8.0, 200 mM NaCl, 1% sodium dodecyl sulfate, 0.2 mg/mL glycogen.
13. PE buffer (2X): 100 mM Tris-HCl (pH 8.3 at 42°C), 100 mM KCl, 20 mM MgCl_2 , 20 mM DTT, 2 mM dNTPs, 1 mM spermidine.
14. TBE (10X): 1 M Tris base, 900 mM boric acid, 10 mM EDTA.
15. Formamide loading mix: 98% formamide, 10 mM EDTA (pH 8.0), 0.01% xylene cyanol, 0.01% bromophenol blue.
16. Acrylamide gel mix: 40% acrylamide/*bis*-acrylamide (29:1).
17. Corning polypropylene centrifuge tubes, 250-mL (cat. no. 25350-250).
18. Corning polypropylene disposable centrifuge tubes, 50-mL (cat. no. 430291).
19. Corning polypropylene disposable centrifuge tubes, 15-mL (cat. no. 430766).
20. Kontes B pestle (VWR).
21. Spectro-Por dialysis tubing (18-mm flat width, molecular-weight [MW] cutoff: 2000) (VWR).
22. Slide-A-Lyzer, 3000 MW cut-off (Pierce).
23. Centiprep YM-3, 3000 MW cut-off (Millipore).
24. Centricon YM-3, 3000 MW cut-off (Millipore).
25. Affi-Gel 10 (Biorad).
26. ^{32}P -ATP (approx 1000 Ci/mmol).

27. T4 DNA ligase.
28. ATP, GTP, CTP, UTP: 5 mM each.
29. 0.3 M Sodium acetate.
30. Phenol:chloroform:isoamyl alcohol (50:50:2), saturated with RNase-free water.
31. 80% Ethanol.
32. 40 mM Sodium pyrophosphate.
33. AMV reverse transcriptase (Promega).
34. Siliconizing reagent: Rain-X (Unelko Corp.).
35. Urea (Invitrogen).
36. 3 MM filter paper sheets.
37. ^{32}P -UTP (800–1000 Ci/mmol).

3. Methods

3.1. Preparation of the Nuclear Transcription Extract

Nuclear extracts can be made from virtually any volume of cells grown in monolayer or in suspension culture (6). For reproducibility and convenience, the majority of nuclear extracts are from liter quantities of HeLa cell suspension culture. The protocol used in our laboratory is a modification of that of Dignam et al. (5). We use suspension cultures of HeLa cells grown in Joklik's minimum essential medium (ICN) supplemented with 10% calf serum.

1. Six liters of HeLa cells in suspension are grown by feeding cells every day while maintaining a cell density of about 6×10^5 per mL (14). Cells should be harvested for the preparation of nuclear extracts when they are growing well, i.e., doubling nearly every 24 h.
2. Harvest cells in six 250-mL Corning polypropylene centrifuge tubes by repeated centrifugation at 4°C for 10 min at 180g (Beckman J6-HC centrifuge), i.e., carefully pour off the supernatant and add fresh suspension culture on top of the existing cell pellet and repeat centrifugation until the entire 6 L of suspension culture are harvested.
3. All subsequent steps are done at 4°C using precooled buffers. Gently resuspend cell pellets into 40 mL of PBS by pipetting, combine the suspended pellets in a single 50-mL Corning polypropylene centrifuge tube, and centrifuge again. Gently resuspend the packed cell pellet (usually 5–8 mL; note exact volume) in five times the pellet volume of hypotonic buffer A and incubate on ice for 10 min. Centrifuge the swollen cells for 10 min at 180g and carefully withdraw the supernatant with a pipet so as not to disturb the soft, swollen cell pellet. Using buffer A, resuspend the cells in twice the *original* volume of packed cells. A microscopic examination of a small aliquot should reveal that cells are greatly swollen but largely intact.
4. Dounce-homogenize the cells using 10 strokes of a Kontes B pestle. A microscopic examination should show that more than 90% of the cells have been disrupted and that the vast majority of nuclei are intact.

5. Centrifuge the nuclear preparation in a 50-mL polypropylene disposable centrifuge tube at 1000g for 10 min (Beckman Avanti J-E centrifuge using a JA-12 rotor). Remove the supernatant and recentrifuge at 1000g for 5 min. Remove and discard the small amount of remaining supernatant above the nuclear pellet. Note the volume of the nuclear pellet.
6. To prepare the nuclear extract, resuspend the nuclear pellet in a volume of low-salt buffer representing *exactly* one-half the nuclear pellet volume. Add dropwise the same volume of high-salt buffer as low-salt buffer while gently vortexing the tube. Tightly cap the tube and continue to extract the pellet by rotation for 30 min.
7. Centrifuge the extract at 9000g for 30 min (Beckman Avanti J-E centrifuge using a JA-12 rotor). Transfer the supernatant into Spectra-Por dialysis tubing and dialyze for 3–5 h against 1 L of buffer D.
8. Remove the extract from the dialysis tubing and clarify it by centrifugation for 10 min at 10,000g. Aliquot the supernatant (100–500 μ L) into precooled microfuge tubes (4°C) and freeze by immersion in liquid nitrogen. Store in a –70°C freezer or in a vapor-phase nitrogen freezer for long-term storage.

3.2. Preparation of Biologically Active E1A Functional Domains

1. Biologically functional His6-tagged E1A 243R and E1A 1-80 polypeptides are prepared by a protocol modified from that suggested by QIAGEN (QIA expressionist). Harvest a culture of isopropylthio- β -D-galactoside-induced bacterial cells (500 mL) expressing an appropriate pQE (QIAGEN) E1A construct (**I2**) by centrifugation at 2000g for 10 min at 4°C (Beckman J6-HC centrifuge), and freeze the cell pellet at –20°C.
2. Thaw the pellet at room temperature and lyse in 40 mL of QIAGEN buffer A with gentle mixing for 1 h. Clarify the supernatant by centrifugation at 9000g for 30 min at 4°C (Beckman Avanti J-E centrifuge; JA-12 rotor).
3. Bind the His6-tagged polypeptide to 2 mL of Ni-NTA resin (QIAGEN) by rotating the clarified lysate with the resin overnight at 4°C. Batch wash the resin five times with 20 mL of buffer A and five times with buffer A adjusted to pH 6.3.
4. Load the resin into two 5-mL columns (Image Molding) and wash each column with 20 mL (10 column volumes) of the same buffer and then with 50 mL of buffer A adjusted to pH 5.9.
5. Elute the His6-tagged polypeptide from the resin with 20 mL of buffer A adjusted to pH 4.5. Collect 2-mL fractions. Generally, sufficient polypeptide is produced so that protein-containing fractions can be identified by adding 2 μ L of each fraction to 100 μ L of BIO-RAD DC Protein Assay Reagent B. Detectable color develops after 10 min at room temperature.
6. To prepare biologically active E1A 1-80 polypeptides, it is necessary to remove guanidine-HCl slowly from the preparation to facilitate proper folding. Pool fractions containing eluted polypeptide and adjust to 6 mL with elution buffer (buffer A at pH 4.5 containing 6 M guanidine-HCl). Dilute the sample 1:1 with 0.5X buffer D.

7. Dialyze the diluted sample (now at 3 M guanidine-HCl) in a 3000 MW cutoff Slide-A-Lyzer dialysis cassette against 0.5X buffer D containing 2 M guanidine-HCl. After 6–8 h of dialysis, remove one-half of the dialysis buffer and replace it with fresh 0.5X buffer D without guanidine-HCl, thereby reducing the guanidine-HCl concentration by half.
8. Continue dialysis with buffer replacement in the manner described above until the guanidine-HCl concentration is reduced to 50–100 mM. Complete the dialysis against several changes of 0.5X buffer D for 8 h.
9. Concentrate the E1A polypeptides by size exclusion centrifugation using Centriprep YM-3 followed by Centricon YM-3 to a final concentration of about 1 mg per mL of polypeptide.

3.3. Preparation of Nuclear Transcription Extracts Affinity Depleted With E1A Functional Domain Polypeptides

1. Affi-Gel 10 immobilized E1A polypeptides are prepared as follows. Exchange by dialysis into 20 mM HEPES, pH 7.2, the E1A 1-80 polypeptides purified as described above (**Subheading 3.2.**).
2. Prepare 1.5 mL of packed Affi-Gel 10 beads immediately prior to use as follows. Take 3 mL of Affi-Gel suspension (comes as a 1:1 slurry in isopropanol) and centrifuge in a 15-mL centrifuge tube (Corning) at 2000 rpm (1000g) for 5 min. Wash beads three times with 12 mL of cold water by centrifugation at 2500 rpm (1700g) for 2 min. Incubate the packed beads with 1.5 mg of polypeptide in 1.5 mL of buffer for 4 h at 4°C with rotation. Retain 10 µL of polypeptide prior to addition to beads and after 4 h of incubation to determine the efficiency of polypeptide binding as follows.
3. Centrifuge the aliquots in a microfuge tube at 1700g for 5 min and add 5 µL of 1 N HCl to the supernatant. Add 40 µL of water and read the absorbance at 280 nm. Efficient binding is reflected by a reduction in absorbance at 280 nm of 80–90%. If efficient binding is not attained, continue rotation of the beads with the polypeptide.
4. When satisfactory binding of polypeptide to beads is obtained, centrifuge the beads at 2000 rpm for 5 min and resuspend the beads in 800 µL of PBS. Add 200 µL of 1 M ethanolamine, pH 8.0, and rotate for 1 h at 4°C to block reactive groups remaining on the beads. Centrifuge to remove the ethanolamine and wash three times with buffer D. Store the polypeptide-immobilized beads at 4°C.
5. Prepare affinity column (5-mL, Image Molding) by loading 250 µL of the packed polypeptide-immobilized beads. Recirculate 1 mL of HeLa cell nuclear extract through each column for 2 h at 4°C at a flow rate of 0.1 mL per min. Aliquots of the nuclear extract before and after affinity chromatography (as well as the proteins bound to the beads) can be analyzed by immunoblot analysis with specific antibodies to candidate E1A cellular partners by use of a sensitive Western blotting kits utilizing chemiluminescent substrates (Amersham Pharmacia, Pierce). The depleted nuclear extracts can now be used by *in vitro* transcription to probe the function of the E1A polypeptide interacting domain(s).

3.4. Analysis of In Vitro Transcription by Primer Extension and by Run-Off Assay

Inasmuch as RNA polymerase II does not efficiently terminate transcription in vitro, two assays have been developed that yield products of discrete length. The first assay is the run-off assay, which uses a DNA template cut with a restriction enzyme downstream of the transcription start site. This simple solution creates a site where the polymerase will fall off the template, thus terminating transcription. By use of a ^{32}P -labeled rNTP precursor, an RNA transcription product of specific length is synthesized and is resolved as a discrete band by denaturing polyacrylamide gel electrophoresis (PAGE) followed by autoradiography or phosphor image analysis. The second assay is primer extension in which the RNA product of the in vitro transcription reaction is annealed with a ^{32}P -end labeled deoxyoligonucleotide complementary to a discrete sequence downstream of the transcription start site. Reverse transcription of the hybrid RNA product/DNA primer yields a labeled DNA fragment of discrete length that spans the sequence between the 5'-end of the primer and the transcription start site. This labeled cDNA fragment is resolved by denaturing PAGE followed by autoradiography. The product of both assays can be quantitated by scanning densitometry or by more sensitive phosphor image analysis.

In general, primer extension is more sensitive than run-off assay and permits a more accurate measurement of the transcription product from the authentic transcription start site. For many studies, a sequence that is specific for a common reporter gene, such as CAT or luciferase, allows the convenient use of a single primer downstream of a variety of promoters for primer extension analysis. This also permits the use of multiple DNA template promoters in a single transcription reaction because the 5'-transcribed sequence from each promoter is usually sufficiently different in size and thus gives rise to primer extension products of different lengths, which can be visualized on a single lane of a gel.

3.4.1. Preparation of DNA Templates for In Vitro Transcription

Plasmid templates containing the promoter of interest must be of high purity for in vitro transcription. Plasmids purified by double CsCl density gradient centrifugation are of high quality and can be used directly. Plasmid templates prepared by standard alkaline lysis procedures or by use of commercial plasmid preparation kits may require further purification. It is often important to further purify those templates by phenol-chloroform extraction followed by ethanol precipitation. For primer extension, superhelical plasmid templates are used. For run-off analysis, templates are linearized with a restriction enzyme in order to terminate transcription at a known site.

3.4.2. Preparation of Radiolabeled Deoxyoligonucleotides for Primer Extension

Deoxyoligonucleotide primers are designed to be about 30 nucleotides (nt) in length and to be complementary to a region from 100 to 200 nt downstream of the transcription initiation site. Primers with self-complementary sequences are avoided. To label a primer, incubate 10 pmol of deoxyoligonucleotide at 37°C for 30 min in a 10-μL reaction containing 1 μL of 10X forward exchange buffer, 6 μL of $\gamma^{32}\text{P}$ -ATP (1000 Ci/mmol), and 1 μL of T4 polynucleotide kinase (10 U). Heat the reaction mixture at 100°C for 2 min and add 190 μL of water. Store at -20°C. The radiolabeled primer may be used as long as a suitable transcription signal is obtained, usually 4–6 wk.

3.4.3. In Vitro Transcription Analysis Using Primer Extension Analysis

3.4.3.1. TRANSCRIPTION REACTION

Reaction mixtures are assembled in RNase-free microfuge tubes containing 2.5 μL of 10X transcription buffer, 2.5 μL of rNTP mixture (5 mM each of ATP, GTP, CTP, and UTP), DNA template (typically 500 ng), nuclear extract (typically 10 μL), and water to give a final volume of 25 μL. The reaction is initiated by the addition of nuclear extract followed by incubation at 30°C for 60 min. For each new DNA template and for each batch of nuclear extract, preliminary titrations are done with different amounts of template (100–1000 ng) and nuclear extract (5.0–12.5 μL) to optimize the transcription signal (strength and authenticity, i.e., correct size). Compensate for volumes of nuclear extract less than 10 μL by the addition of an equivalent volume of buffer D.

3.4.3.2. ISOLATION OF THE RNA TRANSCRIPTION PRODUCT

When the transcription reaction is completed after 60 min, terminate the reaction by addition of 100 μL of stop mix. Then add 300 μL of 0.3 M sodium acetate and 300 μL of phenol/chloroform/isoamyl alcohol (50:50:2). Vortex the sample for 30 s and separate the phases by centrifugation at 10,000g for 2 min. Transfer the upper aqueous phase to a fresh tube containing 1 mL of ethanol, mix, and place on dry ice for 15 min. Centrifuge the sample at 10,000g for 10 min at 4°C, rinse the pellet carefully with 500 μL of 80% ethanol (-20°C), and briefly dry the pellet containing the RNA product in a vacuum dessicator.

3.4.3.3. PRIMER EXTENSION ANALYSIS

Resuspend the RNA pellet completely in 5 μL of 2X PE buffer, 5 μL of water, and 1 μL of the labeled primer (~0.5 pmol) by repeated vortexing and centrifugation. Denature the RNA by incubation at 65°C for 10 min and anneal

the RNA with the primer by incubation at 42°C for 10 min. Next add 5 μ L of 2X PE buffer, 1.6 μ L of water, 1.4 μ L of 40 mM sodium pyrophosphate, 1 μ L of AMV reverse transcriptase (2.5 units, Promega) and incubate the mixture at 42°C for 30 min. Terminate the reverse transcriptase reaction by the addition of 20 μ L of formamide loading mix.

3.4.3.4. RESOLUTION OF THE LABELED PRIMER EXTENSION PRODUCT BY DENATURING POLYACRYLAMIDE GEL

Resolve the labeled product by electrophoresis on a 0.75-mm-thick/20-cm-long 6% urea polyacrylamide gel using a vertical gel chamber. The smaller plate (facing the apparatus) is siliconized with Rain-X to facilitate separation of the plates after electrophoresis. To prepare the gel, stir with a magnetic bar 12 g of urea (ultrapure, RNase-free) with 6.0 mL of 5X TBE, 4.5 mL of 40% acrylamide/*bis*-acrylamide (29:1), 300 μ L of 10% ammonium persulfate, and water to a final volume of 30 mL. When the urea is completely dissolved, add 20 μ L of TEMED and pipet or pour the mixture into an assembled gel sandwich held at about a 40° angle. Insert a 15-well comb and then allow the gel to polymerize in a horizontal position for 3 h or overnight. Prior to loading the samples, mount the gel on the electrophoresis chamber, add TBE to the upper and lower chambers, and clean the gel teeth by pipetting TBE up and down. Pre-electrophorese the gel for about 15 min at 400 V constant. Load the samples (10–20 μ L) and a labeled size marker (e.g., a 50-bp ladder; *see Note 2*) into individual lanes on the gel and electrophorese at 400 V constant until the bromophenol blue marker just migrates off the gel.

The gel is pulled onto 3MM paper as follows. Briefly, separate the gel sandwich by gentle prying with a fine-bladed spatula. The gel will adhere to the larger, unsiliconized plate. Put the gel-containing plate flat on a bench top and carefully position a sheet of 3MM paper over the gel. Rub the paper gently but firmly and carefully pull off the paper with the adherent gel. The gel can then be dried on a gel dryer prior to autoradiography on X-ray film or simply covered with plastic wrap and autoradiographed with X-ray film and an intensifying screen at –70°C overnight. For quantitation and highly sensitive detection of signals, gels are dried and analyzed by phosphor image analysis as described in **Subheading 3.4.5**.

3.4.4. *In Vitro* Transcription Using the Run-Off Assay

The transcription reaction for the run-off assay is virtually the same as that described in **Subheading 3.4.3.1** with the following modifications. First, the DNA template is cut at a convenient site with a restriction enzyme. Second, the 10X rNTP mixture consists of 5 mM ATP, GTP, and CTP but only 0.25 mM UTP. Third, ³²P-UTP (0.5 μ L of 10 mCi/mL, 800–1000 Ci/mmol) is added to

the reaction mixture. Finally, RNA is isolated as described in **Subheading 3.4.3.2.** and is directly analyzed by gel electrophoresis as described in **Subheading 3.4.3.4.**

3.4.5. Phosphor Image Analysis and Quantitation of Transcription Products From Primer Extension and Run-Off Assays

Regulatory proteins and mutants of those proteins may activate or repress transcription at varying rates as opposed to an all-or-none phenomenon. Multiple titrations with different levels of polypeptides followed by careful quantitation of transcription product are important to assess function. Phosphor image analysis provides for an efficient, accurate, and highly sensitive way to measure radiolabeled transcription products. Phosphor image analysis typically has a linear dynamic range of 5 orders of magnitude as compared to only about 1.5 for X-ray film. The pixels measured by the PhosphorImager can be quantitatively and statistically analyzed using software provided by the manufacturer, e.g., Image Quant for the Molecular Dynamics PhosphorImager system used here.

Briefly, cover the dried gel with plastic wrap and place a phosphor screen over the dry gel in a PhosphorImager cassette. Expose the phosphor screen from overnight to 2 d. Place the screen face-down on the PhosphorImager glass plate and select the scanning area using the appropriate software. The image of the gel is visualized and bands of the correct size are selected and quantified using the tools provided by the PhosphorImager software. These images can be printed or saved as digital files for permanent storage and for publication.

4. Notes

1. It is important that the transcription signal be optimized prior to assessing the effects of added regulatory protein. In the case of transcriptional activation, it is important to use conditions that are suboptimal in the absence of added activator so that transactivation can be easily measured over a wide dynamic range. By contrast, when analyzing transcription repression, it is desirable to use conditions that provide a moderate transcription signal, thus providing a sensitive assay for measuring repression by added protein. These goals can often be achieved empirically by one of two means. First, the amount of nuclear extract in the reaction mixture can often be titrated to provide the desired level of transcription in the absence of added protein factor. Second, it is possible to prepare extracts that possess innately high or low activity by altering the ionic strength of the salt used to prepare the nuclear extract. These extracts may contain greater or lesser amounts of transcription factors or cellular inhibitors of transcription, some of which may represent a cellular target of the added regulatory protein. The putative cellular target may not be rate-limiting in the nuclear transcription extract prepared under standard conditions. Specific modifications of the reaction mix-

ture can also achieve the desired results. For example, to demonstrate in vitro transactivation of the HIV LTR promoter by the HIV-1 Tat transactivator protein in run-off assays, it was necessary to reduce the basal transcription level of extracts by the addition of 6 mM sodium citrate (15).

2. As a size marker for analysis of transcription products, it is convenient to end-label a 50- or 100-bp DNA ladder as described by the manufacturer (Invitrogen).

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References

1. Shenk, T. (2001) *Adenoviridae*: the viruses and their replication, in *Fundamental Virology*, Lippincott, Williams and Wilkins, New York, pp. 1053–1088.
2. Chinnadurai, G. (2002) CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol. Cell* **9**, 213–224.
3. Song, C.-Z., Tierney, C. J., Loewenstein, P. M., et al. (1995) Transcriptional repression by human adenovirus E1A N-terminus/conserved domain 1 polypeptides in vivo and in vitro in the absence of protein synthesis. *J. Biol. Chem.* **40**, 23,263–23,267.
4. Roeder, R. G. (2005) Transcriptional regulation and the role of diverse coactivators in animal cells. *FEBS Lett.* **579**, 909–915.
5. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucl. Acids Res.* **11**, 1475–1489.
6. Lee, K. A. W., and Green, M. R. (1990) Small-scale preparation of extracts from radiolabeled cells efficient in pre-mRNA splicing. *Methods Enzymol.* **181**, 20–30.
7. Lusser, A., and Kadonaga, J. T. (2004) Strategies for the reconstitution of chromatin. *Nat. Methods* **1**, 19–26.
8. Jenuwein, T., and Allis, C. D. (2001) Translating the histone code. *Science* **293**, 1074–1080.
9. Fyodorov, D. V., and Kadonaga, J. T. (2003) Chromatin assembly in vitro with purified recombinant ACF and NAP-1. *Methods Enzymol.* **371**, 499–515.
10. Green, M., Loewenstein, P. M., Pusztai, R., and Symington, J. S. (1988) An adenovirus E1A protein domain activates transcription in vivo and in vitro in the absence of protein synthesis. *Cell* **53**, 921–926.
11. Tang, H., Sun, X., Reinberg, D., and Ebright, R. H. (1996) Protein-protein interactions in eukaryotic transcription initiation: structure of the preinitiation complex. *Proc. Natl. Acad. Sci. USA* **93**, 1119–1124.
12. Boyd, J. M., Loewenstein, P. M., Tang, Q.-Q., Yu, L., and Green, M. (2002) Adenovirus E1A N-terminal amino acid sequence requirements for repression of

transcription in vitro and in vivo correlate with those required for E1A interference with TBP-TATA complex formation. *J. Virol.* **76**, 1461–1474.

13. Song, C.-Z., Loewenstein, P. M., Toth, K., and Green, M. (1995) TFIID is a direct functional target of the adenovirus E1A transcription-repression domain. *Proc. Natl. Acad. Sci. USA* **92**, 10,330–10,333.
14. Green, M. and Loewenstein, P. M. (2005) Human adenoviruses: propagation, purification, quantification, and storage, in *Current Protocols in Microbiology*, John Wiley and Sons, Inc, New York, pp. 14C.1.1.–14C.1.9.
15. Kato, H., Sumimoto, H., Pognonec, T., Chen, C.-H., Rosen, C. A., and Roeder, R. G. (1992) HIV-1 TaT acts as a processivity factor in vitro in conjunction with cellular elongation factors. *Gene Dev.* **6**, 655–666.

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