Purification and Biological Characterization of an Adenovirus Type 2 E1A Protein Expressed in Escherichia coli

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The adenovirus 2 E1A gene encodes a multifunctional protein of 289 amino acids that can immortalize primary rodent cells and transcriptionally activate a number of viral and cellular genes. To facilitate an understanding of the molecular basis for the various actions of E1A, we have redesigned our bacterial expression vector (Ko, J.-L., and Harter, M. L. (1984) Mol. Cell. Biol. 4, 1427–1439) containing the cloned E1A gene such that a soluble authentic E1A protein now constitutes approximately 1.5% of the total Escherichia coli cellular protein. Further, we have developed a simple rapid purification scheme without the use of detergents or denaturants and show a purity of >98% with a yield of approximately 53%. The E1A so purified is biologically active, stimulating cellular DNA synthesis following microinjection into quiescent NIH 3T3 and REF52 cells. In another report (Spangler, R., Bruner, M., Dalie, B., and Harter, M. L. (1987) Science 237, 1044–1046) we have also shown that our purified E1A protein activates transcription from appropriate promoters in an in vitro system.

The E1A protein encoded by the Ad2\(^1\) E1A gene is a polypeptide of 289 amino acids that has been shown by mutational analysis to be composed of at least three functional domains (for review, see Refs. 1 and 2). Each of these domains appears to be capable of functioning independently, and each has been shown to be associated with a biochemical activity that is sufficient to either regulate RNA transcription (3), induce cellular DNA synthesis (4, 5), or immortalize primary rat cells in tissue culture (6, 7).

The mechanisms by which the E1A protein operates to fulfill its biological functions are still poorly understood, particularly at the molecular level. Therefore, the availability of this protein in a highly purified form would be quite useful to study its molecular details both in vivo and in vitro. A purified Ad2-Ad5 hybrid E1A-like protein expressed in Escherichia coli (8) has in fact shown some biological activity in vivo. However, this protein was found to be produced in an insoluble form and required the use of strong denaturants for its solubilization. Under these circumstances, the physical condition of the hybrid E1A-like protein might prove to be unacceptable for analysis of its protein structure and function.

We have previously cloned an authentic Ad2 E1A gene in a bacterial plasmid (pKHAO) and have demonstrated its expression in E. coli (9). Moreover, the E1A protein produced in this system was found to be of the exact length and correct amino acid sequence as that synthesized in an Ad2-infected cell. Here we describe the use of a new plasmid expression vector (pKHAO-T) that allows for the efficient expression and purification of our E1A protein in bacterial cells. The 289-amino acid E1A protein is soluble upon cell lysis (constituting approximately 1.0–1.5% of the total E. coli protein) and most importantly, it is purified without the use of detergents or chaotropic agents. We also present evidence that our purified E1A protein microinjected into quiescent NIH 3T3 or REF52 cells is capable of inducing cellular DNA synthesis and is, therefore, biologically active. Furthermore, we have demonstrated that this E1A protein is also functionally active in an in vitro transcriptional system as described elsewhere (10).

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Expression of the E1A Protein in Bacteria—We have previously reported (9) the construction of a vector (pKHAO) that produces in E. coli a corresponding adenovirus E1A protein of exact length (289R) and putative correct amino acid sequence as that synthesized in an adenovirus-infected cell. In that plasmid, transcription, of the E1A protein was directed by the lacUV5 promoter, and the amount of E1A ultimately produced in conventional strains of E. coli constituted approximately 0.1–0.3% of the total cellular protein (9).

In order to increase the production of E1A in bacteria, we converted the lacUV5 promoter in the pKHAO plasmid to a promoter (lac) consisting of the tryptophan promoter and the lac operator and Shine-Dalgarno sequence. This hybrid promoter has already been shown to be significantly more efficient than the lac promoter (15), and like its predecessor, it can be induced to full strength by the lac inducer, isopropyl-\(\beta\)-D-thiogalactopyranoside (details of construction of the new E1A expression vector, designated pKHAO-T, are given under "Experimental Procedures").
The production of the E1A protein in bacterial cells bearing the pKHAO-T plasmid minus the lacI gene was initially demonstrated in strains of E. coli carrying the lacI allele on the chromosome. Although the overall production of E1A in these bacterial cells appeared to increase, the bacterial response in allowing this protein to remain stable was far less than we had anticipated (data not shown). We decided then to transfer the pKHAO-T plasmid carrying the lacI gene to a number of lon" strains (protease-deficient mutants) in the hope of finding a bacterial environment that would confer a lower rate of E1A degradation. To determine whether E1A could accumulate to high levels in any of those mutants, cultures of cells either with or without pKHAO-T were pulse-labeled with [35S]methionine after isopropyl-β-D-thiogalactopyranoside induction and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. One mutant in particular, namely MC102, produced a 45-kDa protein (Fig. 2, lane 1) of considerable amount which was noticeably absent in control cells lacking the pKHAO-T plasmid (Fig. 2, lane 4). Moreover, this protein was of the same size as that previously shown to be synthesized by the original expression vector (pKHAO) when in maxicells (9) and E. coli S-50 extracts (20). We next used an affinity-purified E1A-specific monoclonal antibody, M-2 (11), to confirm the antigenicity of the pKHAO-T plasmid-encoded protein. As shown in Fig. 2, lane 2, the M-2 antibody selectively precipitated a fully intact 35S-labeled protein of 45 kDa from the extracts of MC102 cells containing the pKHAO-T plasmid. Extracts of MC102 cells without pKHAO-T produced no observable 35S-labeled products when immunoprecipitated with the same antibody (Fig. 2, lane 5), and the 45-kDa protein was not precipitated from extracts of cells either with or without the pKHAO-T plasmid when the control antibody was used (Fig. 2, lanes 3 and 6, respectively). We also examined the effects of other E1A-specific monoclonal antibodies, namely M-1, M-3, and M-73 (11), and all of these behaved similar to M-2 in that each one of them identified a 45-kDa protein only in those cells that carried the pKHAO-T plasmid (data not shown). We can safely conclude then that the modified vector, pKHAO-T, like its predecessor, encodes an authentic E1A protein, and when synthesized in a lon" system the protein appears to remain relatively unaffected by proteolysis.

**Purification of the E1A Protein**—The purification procedures that we found to be most useful for isolating a biologically active E1A protein are summarized in Table I. This relatively simple and rapid method for purifying E1A was developed with the help of a dot-immunobinding assay (see "Experimental Procedures") that included the use of the three E1A-specific monoclonal antibodies described above. The assay was routinely performed in triplicate after each step of the purification, and this enabled us then to monitor the protein at all times.

The most important aspect of the purification is that the E1A protein is found almost exclusively in the soluble portion of the cell extract and, therefore, did not require at any time the use of strong denaturants for its recovery. The details of the purification of E1A are given under "Experimental Procedures," but briefly, proteins in the extracts of MC102 cells were first fractionated by precipitation with ammonium sulfate. After assaying with E1A-specific antibody, we found that E1A precipitated in the 15–40% ammonium sulfate fraction with a yield of 98% (Table I). The partially purified protein was then dialyzed extensively to lower the pH to 5.9 and afterwards applied to a CM-Sepharose CL-6B column. Fig. 3 shows the pattern of the distributed E. coli proteins as a function of absorbance after stepwise elution with KCl in buffer and also shows the locations of the E1A protein (fractions under the arrow) as determined by dot-immunobinding analysis. Almost all of the contaminating protein (96%) and at least 39% of the total amount of E1A protein passed through the column without binding (Table I; Fig. 3, fractions 1–20). The remainder of the E1A protein was eluted at salt concentrations of 0.15 M (Fig. 3, fractions 71–78) and 0.3 M KCl (Fig. 3, fractions 90–97) with calculated yields of 6.2 and 32.5%, respectively (Table I). A sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the E1A-containing fractions resulting from each step of the purification is shown in Fig. 4A. Unquestionably, the most purified form of the E1A protein can be found in the 0.3 M eluate fractions derived from the CM-Sepharose column (Fig. 4A, lane 5), and judging from the gel scan (data not shown), the purity was more than 98%, even when 5 µg of purified E1A was loaded onto the gel. As expected, the M-2 monoclonal antibody readily identified by immunoblot analysis the purified form of the E1A protein without the appearance of proteolytic products (Fig. 4B, lane 1). The CM-Sepharose column then clearly enriches for the E1A protein and more importantly does so under elution conditions which favor high yield both in sample mass (approximately 1 mg/µl of bacterial culture) and biological activity.

**Microinjection of Purified E1A into Quiescent Cells**—It has been previously shown that a microinjected plasmid encoding the E1A protein can stimulate cellular DNA synthesis in quiescent cells (5). We, therefore, decided to test by microinjection whether our purified E1A could also induce cellular DNA synthesis in quiescent cells. A fundamental way to assay for DNA synthesis is to label growth-arrested cells with [3H] thymidine after microinjection and afterwards screen for the incorporation of radioactive into cell nuclei by autoradiography. For our study we examined two different established cell lines. As shown in Table II, the purified E1A protein is able to stimulate cellular DNA synthesis when microinjected into quiescent mouse NIH 3T3 or rat REF52 cells. Compared to the injection of bovine serum albumin which had no effect on DNA synthesis, purified E1A protein at its highest concentration (0.8 mg/ml) triggered DNA synthesis in about 36% of the REF52 cells and 18% of the NIH 3T3 cells. Addition of pser platelet-derived growth factor or 10% fetal calf serum to both of the arrested cell lines induced DNA synthesis in 90% of the cells (data not shown). Collectively, these results show that our purified E1A protein is biologically active, and its efficiency in stimulating cellular DNA synthesis appears to depend on the cell type examined.

**DISCUSSION**

We have maximized the expression of an authentic Ad2 E1A protein in E. coli by converting the lacUV5 promoter on our former expression vector pKHAO (9) to a promoter (tac) of greater strength (15). The decision to use the tac promoter instead of the λp, or trp promoter for controlling the expression of E1A was to prevent overproduction of the protein to an insoluble form. Indeed, proteins produced in an insoluble form always require the use of strong denaturants for their solubilization, and treatment of this type usually proves unacceptable for detailed analysis of a protein in an in vitro environment. As demonstrated under "Results," intracellular aggregation of the E1A protein does not appear to be the case, and when the tac promoter on the new plasmid vector (pKHAO-T) functions in a lon" strain, E1A is synthesized in an amount that approximates 1.2–1.5% of the total cellular protein. The E1A protein also appears to be quite stable in the lon" mutant: proteolytic products do not appear after
purifying E1A with a number of E1A-specific monoclonal antibodies (11).

A protein that is soluble in bacteria can usually be recovered by extractions with neutral aqueous buffer. Such was the case for our E. coli-produced E1A protein, and without the assistance of harsh eluants the protein was purified to approximately 98% homogeneity. The purification procedure described in this paper for E1A is relatively rapid and simple. The first step of the procedure involves an ammonium sulfate fractionation which removes over 62% of the endogenous protein with full recovery of the E1A protein in the 15–40% cut. In the next and final step, E1A is enriched by passage of the 15–40% fraction over a CM-Sepharose column. The flow-through and the 0.15 M eluate fractions from the column remove virtually all of the protein contaminants, and the remaining 0.3 M fractions contain almost exclusively E1A with an overall yield of 93%. It is interesting that a portion of the E1A (39%) passes through the column without initially binding to the CM-Sepharose matrix. This finding is reminiscent of our previous work (20) involving the study of an unpurified preparation of our E1A on a variety of DNA-cellulose columns. In these experiments, E1A would invariably exhibit an overall yield of 53%. It is interesting that a portion of our earlier work (20) involving the study of an unpurified preparation of our E1A on a variety of DNA-cellulose columns, or alternatively E1A could be interacting with itself, in both of these situations E1A may be interacting with an upstream viral enhancer. Currently, we are investigating the nature of this repression by E1A and its role in possibly displacing a cellular factor that may be binding to the enhancer region for RNA stimulation.

The mechanism by which the Ad2 E1A protein operates is still poorly understood. Our ability to efficiently express and purify an authentic E1A protein that is biologically active provides an opportunity for us to study in detail the physical and biochemical parameters associated with this protein.

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S. Datta and M. L. Harter, unpublished observations.
**EXPERIMENTAL PROCEDURES**

**Cell Culture**: NIH 3T3 mouse and REF52 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum until they reached less than full confluency. To make the cells go through for microinjection, 4 x 10^5 fetal calf serum. After 24 h the medium was changed to DMEM supplemented with 2.5% fetal calf serum. After another 36-48 h, the cells were used for microinjection.

**Table I**: Purification of EIA protein from MCl02a

| Step                  | Total  | Total EIA  | EIA yield  |
|-----------------------|--------|------------|------------|
| 1. Crude extract      | 10^6   | 12.0       | 1.2        |
| 2. Ammonium sulfate fraction | 384    | 11.8       | 98.0       |
| 3. CM-Sepharose flow-through | 333    | 1.2        | 36.0       |
| 4. CM-Sepharose pooled 0.15 M KCl | 66     | 0.9        | 6.7        |
| 5. CM-Sepharose pooled 0.3 M KCl | 65     | 6.3        | 98.3       |

**Macrophage Culture**: NIH 3T3 and REF52 cells were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum until they reached less than 90% confluence. 10^5 cells per 0.2 ml were seeded into 1.5 ml in 10-mm dishes containing 1% fetal calf serum. After the cells were harvested, they were suspended in 45 μl of Laemmli loading buffer, boiled for 10 min, and then loaded onto a 12% SDS-polyacrylamide gel. The gel was run under standard conditions in an electrophoresis apparatus.

**Table II**: Inhibition of DNA Synthesis After Microinjection of EIA

| Protein concentrate | Percentage of labeled cells |
|---------------------|----------------------------|
| 0.6 mg/ml            | 62 ± 18                    |
| 0.8 mg/ml            | 62 ± 18                    |
| 1.0 mg/ml            | 62 ± 18                    |
| 2.0 mg/ml            | 62 ± 18                    |
| 3.0 mg/ml            | 62 ± 18                    |

**Notes**

1. *CM-Sepharose CL-6B Chromatography*: Supernatant from above was applied directly to a CM-Sepharose CL-6B column (2 x 4 cm, 6.3 ml for an 80 mg preparation) previously equilibrated with Buffer C. The column was then washed with an additional 10 ml of Buffer C and protein was eluted with a linear 0-0.5 M NaCl gradient in Buffer C at a flow rate of 15 ml/h. Fractions containing pooled EIA were dialyzed against Buffer D and then stored at -20°C.

2. **Assays**

   **DNA-Immobilizing Assay**: The DNA-immobilizing activity was determined by the following procedure. Cells were grown in 15 ml of Eagle's minimal essential medium (EMEM) containing 10% fetal calf serum and 1% streptomycin-penicillin. The cells were harvested by centrifugation and then resuspended in 1 ml of 0.1 M NaCl containing 0.01 M Na2EDTA, pH 7.4. After DNA was precipitated with 1 ml of ice-cold ethanol, the pellets were washed with 1 ml of 0.1 M NaCl containing 0.01 M Na2EDTA, pH 7.4.

   **Immunoassay**: Cells were harvested, washed, and resuspended in ice-cold 0.1 M NaCl containing 0.01 M Na2EDTA, pH 7.4. Equal volumes of the cells and normal rabbit serum were mixed, and the mixture was incubated at 37°C for 30 min. After being centrifuged, the supernatant was discarded, and the cells were resuspended in 0.1 M NaCl containing 0.01 M Na2EDTA, pH 7.4. Immunofluorescence labeling of cells and autoradiography were performed as described previously.

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Purification of the Adenovirus E1A Protein Made in E. coli

Figure 1. Construction of pKHAD-T containing the tgr promoter and the E1A coding sequence. Vector plKHAD was first digested with HindIII, then with EcoRI and the 3.6 Kpb EcoRI/EcoRI fragment ligated to the 3.5 Kpb HindIII/HindIII fragment, generating pKHAD-T. Plasmid pE300 was cut with ScaI and then digested to the sticky-ended HindIII fragment to generate pKHAD. Afterwards, the pKHAD-E1A fragment was digested with EcoRI and the fragment containing the tgr promoter was isolated. This fragment was then ligated to the HindIII/HindIII fragment derived from plKHAD. The appropriate recombinant containing the tgr promoter with E1A coding sequence in the correct orientation was identified by cloning the E1A photoreporter gene. This plasmid was designated pKHAD-T. The solid circles indicate the sites to which the ScaI/PvuII promoter or gene fragment is oriented; the dotted part of the arrow represents the tgr promoter. Cross-hatched bars represent the coding sequence for E1A, and the double cross-hatched bars represent the photoreporter gene.

Figure 2. Identification of the pKHAD-T-encoded E1A protein in extracts of McA12 cells with M-2 monoclonal antibody. McA12 cells, with and without the pKHAD-T plasmid, were grown at 37°C in 10% fetal calf serum to an A590 of 0.79 at which time IPTG was added to a final concentration of 3.0 mM. Both cultures after 3 h of induction were subjected to immunoprecipitation reactions, incubated for 2 h, and then blotted for E1A. Blots were then hybridized with the E1A coding sequence digested with HindIII, E1A coding sequence digested with ScaI, and E1A coding sequence digested with HindIII/ScaI, respectively. Afterwards, the samples were rinsed and then rehybridized in Lomazol buffer (19) or prehybridized for immunoprecipitation, as described under "Experimental Procedures." All samples were electrophoresed through a 13% SDS-polyacrylamide gel. Lanes 1 and 2, extracts of McA12 cells with lane 1 and without lane 2) pKHAD-T; lanes 3 and 4, McA12 monoclonal antibody precipitate of McA12 cells with lane 3 and without lane 4) pKHAD-T; lanes 5 and 6, McA12 monoclonal antibody (MAB) precipitate of McA12 cells with lane 5 and without lane 6) pKHAD-T lane M, molecular weight standards.

Figure 3. CM-Sephadex CL-65 chromatography of the E1A protein. A dialyzed 1 M KCl ammonium acetate fraction was applied to a CL-65 column previously equilibrated with 1 M KCl. The column was then eluted with a 300-ml linear gradient of 1 M KCl in 0.2 M KCl in a buffer containing 0.5 M NaCl. The dotted line represents the stepwise elution of the column with increasing KCl. The arrow points to a fraction containing the E1A protein as determined by dot-immunoassay analysis (see "Experimental Procedures").
Purification of the Adenovirus E1A Protein Made in E. coli

Figure 4. SDS-polyacrylamide gel electrophoresis of the purified fractions. Samples derived from each stage of the purification were electrophoresed through a 15% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250 or subjected to immunodot transfer (see "Experimental Procedure") using the M-2 monoclonal antibody 05. Panel A: lane 1, 10 μg of total cellular proteins; lane 2, 1 μg of the 10-40% ammonium sulfate fractions; lane 3, 1 μg of the pooled CM-Sepharose CL-4B flow through; lane 4, 5 μg of the pooled CM-Sepharose CL-4B wash; lane 5, 5 μg of the pooled CM-Sepharose CL-4B elution fractions. Panel B: lane 1, 5 μg of purified E1A proteins; lane M, pre-stained molecular weight markers.