Activation *in vitro* of RNA polymerase II and III directed transcription by baculovirus produced E1A protein

G.Patel and N.C.Jones

Gene Regulation Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK

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ABSTRACT

The baculovirus expression system has been successfully used to overproduce a number of different protein products. In this report we describe the construction of a recombinant baculovirus containing the adenovirus E1A 13s cDNA sequence. Infection of insect cells with this virus results in the production of phosphorylated E1A protein. The phosphorylation pattern appears to be similar to the complex pattern associated with E1A protein synthesis in mammalian cells. Purified baculovirus generated E1A protein activated transcription of specific polIII promoters both in microinjected *Xenopus laevis* oocytes and in HeLa cell *in vitro* transcription extracts. The protein also stimulates *in vitro* transcription of the polIII transcribed VA1 gene.

INTRODUCTION

Regulation of gene expression may be achieved by modulating the binding or activity of sequence-specific DNA binding factors by other trans-acting regulatory proteins. A paradigm for this mode of transcription control is exhibited by the products of the adenovirus E1A gene (for reviews see 1, 2). Transcriptional activation by E1A is a key event in an adenovirus productive infection and at least five viral transcription units require E1A for efficient expression. E1A has also been shown to activate certain cellular genes including those that encode the human 70 kd heat shock protein (3–5) and β -tubulin (6). Remarkably, its stimulatory activity is not restricted to polIII genes since a number of polIII genes including the adenovirus VA genes are also activated (7–11). In contrast to its activation function E1A can also repress a variety of viral and cellular genes by interfering with the ability of their enhancer or promoter elements to stimulate transcription (12–18). The mechanism of either E1A dependent activation or repression remains unknown. E1A could induce an increase in synthesis of certain transcription factors that may be limiting in the cell; however, this possibility is inconsistent with findings showing that some promoters can be activated by E1A *in vitro* implying that no new protein synthesis is required (19, 20). Rather, the results from various studies suggest that E1A regulates transcription by modulating the binding or activity of host cell transcription factors. Such modulation could, for example, be induced directly by protein:protein interaction between E1A and the factors involved

or indirectly by an E1A-induced post-translational modification.

The study of E1A protein *in vitro* will likely be crucial for understandings its mode of action. E1A protein has been overexpressed and purified from bacteria (19, 21) and, at least in one case, shown to have some activity in an *in vitro* transcription system (19). However, the primary translation products of E1A in mammalian cells are extensively modified. This is most vividly demonstrated by high resolution two-dimensional gel electrophoresis where E1A protein made *in vivo* can be resolved into approximately 60 polypeptide species (22). It is clear that most of the heterogeneity is due to differential phosphorylation although other modifications may also play some role. At least seven different phosphorylation sites have been mapped and although many of these sites can be removed or altered without any apparent effect on E1A function (23–26) it is not yet clear whether they are all dispensable. The study of E1A protein *in vitro* would benefit from the availability of large quantities of the modified form of the protein and we therefore explored the helper-independent baculovirus expression system for large scale synthesis of the protein. The system is based on the use of the highly efficient promoter of the polyhedrin gene of the insect baculovirus *Autographa californica* Nuclear Polyhedrosis Virus which is non-essential for infectious extracellular baculovirus production (for reviews see 27, 28). Expression of a number of proteins has revealed that baculovirus-infected *Spodoptera frugiperda* (Sf9) cells can perform many post-translational modifications including phosphorylation (29–32), glycosylation (33–35), accurate signal peptide cleavage (36, 37) and extracellular secretion (36, 37).

Using this system, milligram amounts of E1A protein can be readily synthesised. The protein is extensively phosphorylated and can efficiently activate both RNA polymerase II and III transcription *in vitro*.

MATERIALS AND METHODS

Cells

Insect cell line Sf9 (ATCC accession number CRL 1711) derived from *Spodoptera frugiperda*, was propagated in Grace's medium (Imperial Laboratories) supplemented with 3.3 g/l lactalbumin hydrolysate, 3.3 g/l Yeastolate and 10% foetal calf serum. Sf9 cells were infected in the above medium with the addition of 50 μ g/ml gentamycin sulphate and 2.5 μ g/ml amphotericin (Gibco).

Viral and plasmid DNA

The transfer vector pAc373 and wild type *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV, E2 stock) was obtained from Dr. M. Summers, Texas A&M University. Plasmid pRX3.3 was constructed by replacing the EcoRI-Xba fragment of plasmid JN20 (38), with the EcoRI-Xba fragment from pAS410 (21). pRX3.3 was restricted with NdeI, filled in with Klenow fragment of DNA polymerase, and then subsequently digested with KpnI. The 1.4 Kb cDNA containing the coding sequence of Ad5 E1A (except for the second residue (21)) was transferred into transfer vector pAc373 which had been previously digested with BamHI, filled in with Klenow fragment and later restricted with KpnI. The resulting construct pAc373-13s contains all but eight nucleotides of the polyhedrin 5' untranslated leader sequence fused to Ad5 E1A coding sequences.

Isolation of recombinant baculovirus

Sf9 cells were co-transfected with wild-type AcNPV DNA and the pAc373-13s construct and recombinant baculovirus were isolated as previously described by plaque hybridization (35).

Detection of recombinant protein

Purified recombinant virus was used to infect monolayers of Sf9 cells. At 28–30 hrs p.i. infected, cells were lysed in RIPA buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% Triton, 1% sodium deoxycholate and 0.1% SDS). 293 cells (39) were also lysed in RIPA buffer. Proteins were separated by electrophoresis on SDS polyacrylamide gels and immunoblotted on nitrocellulose. E1A protein was detected with either polyclonal antibody R1 (40) or with monoclonal antibody, M2 (22) using the biotin-streptavidin/system (Amersham).

Immunoprecipitation and 2D-gel electrophoresis

Cell extracts in RIPA buffer were incubated with monoclonal antibody M2 or polyclonal antibody R1 for 1 hr on ice, followed by a 15 min incubation with protein A-sepharose beads. The beads were washed with RIPA buffer, and boiled with Laemmli sample buffer and analysed by one dimensional SDS-PAGE. For two dimensional gel electrophoresis, immunoprecipitations were mixed with sample buffer and electrophoresed essentially as described by O'Farrell (41).

Immunoaffinity chromatography

Infected Sf9 cells were lysed 28 hrs p.i. by the procedure described by Manley (42). Purification of the E1A protein was by chromatography on a protein A-sepharose column containing immobilised M2 monoclonal antibody. The cell lysate was preabsorbed with uncoupled protein A-sepharose to remove non-specific binding and then adsorbed to the column containing the cross-linked monoclonal antibody. The column was washed in sequence with: (i) 0.5 M NaCl, 0.05 M Tris-HCl pH 8.2, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 15% glycerol; (ii) 0.05 M LiCl, 0.05 M Tris-HCl pH 8.2, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 15% glycerol; (iii) 0.05 M NaCl, 0.01 M Tris-HCl pH 8.2, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 15% glycerol. The protein was eluted with 50 mM diethylamine, pH 11.5. 250 μ l fractions were immediately neutralised with 25 μ l of 1 M glycine, pH 2.5. Fractions were assayed for E1A protein by Western blotting and the purity was screened by Coomassie Blue staining of SDS-polyacrylamide gels.

Figure 1. Western blot analysis of E1A protein expressed in mammalian, insect and bacterial cells. Overexpression of E1A protein in *E. coli* was as previously described (21). For synthesis in insect cells, 5×10^4 Sf9 cells were infected with the recombinant baculovirus AcE1A-13s at a moi of 5 pfu/cell. The cells were harvested 28 hours post-infection and lysed in RIPA buffer. For synthesis in mammalian cells, 5×10^6 293 cells which contain an integrated E1A gene, were harvested and lysed in RIPA buffer.

DNA polymerase II-dependent *in vitro* transcription

Transcription reactions were in 50 μ l volume containing 30 μ l HeLa whole cell lysate (BRL Cat. no. 98121), 140 μ M EDTA and 1 mM creatine phosphate. 50 ng purified E1A protein was added to appropriate reactions and the mixture allowed to incubate on ice for 30 min. 1 μ g template DNA (pE2AE-CAT or pTKCAT) was then added and the mixture incubated on ice for a further 30 min. The transcription reaction was started by adding 500 μ M each of ATP, CTP, GTP and UTP and incubated at 30°C for 1 hr. The reaction was stopped by the addition of 50 μ l PK stop (100 mM Tris pH 7.5, 2% SDS, 20 mM EDTA and 400 μ g/ml Proteinase K), and the prepared RNA was hybridised to 32 P-labelled CAT oligo primer DNA for 1 hr at 50°C. The RNA was reverse transcribed with 10 U of AMV reverse transcriptase in the presence of 20 mM Tris pH 8.7, 10 mM MgCl₂, 5 mM DTT and 250 μ M dNTPs and the products separated on a 6% denaturing polyacrylamide gel.

Figure 2. Two-dimensional gel electrophoresis of E1A protein synthesised in infected HeLa and Sf9 cells. HeLa cells were infected with wild type adenovirus at a moi of 20 pfu/cell. Cytosine arabinoside (25 μ g/ml) was added 6 hours post-infection. At 20 hours post-infection, the cells were transferred to methionine-free medium and 35 S-methionine added 30 mins later. Labelling was for 3 hours whereupon the cells were harvested and RIPA extracts prepared. Sf9 cells were infected at 5 pfu/cell with AcE1A-13s. At 26 hours post-infection, the cells were transferred to methionine-free medium and 35 S-methionine added 30 minutes later. The cells were harvested and RIPA-extracts prepared 4 hours later. Two-dimensional gel electrophoresis was as described by O'Farrell (41).

DNA polymerase-III dependent *in vitro* transcription

Whole cell extracts were prepared from HeLa cells by the procedure of Manley *et al.* (42). 15 μ l lysate was assayed in 25 μ l final volume containing 4 mM creatine phosphate, 1 μ g/ml α -amanitin, 600 μ M each ATP, CTP and UTP, 25 μ M GTP and 10 μ Ci [α - 32 P]GTP. The lysate was preincubated at 0°C for 30 mins with purified E1A added at various concentrations ranging from 50 pg to 50 ng per reaction. Plasmid d12 encoding the Ad5 VA1 gene (43) was then added followed 30 mins later by the nucleotide triphosphates to begin the reaction. The mixture was incubated for 1 hr at 30°C and the products analysed on a 6% denaturing polyacrylamide gel. For the antibody inhibition experiments, polyclonal antibody R1 or monoclonal antibody M73 (22) were added to 100 ng E1A protein and incubated on ice for 20 min prior to the addition of HeLa cell lysate, DNA template and triphosphates.

RESULTS

Expression and characterisation of baculovirus produced E1A

During a productive adenovirus infection, the E1A gene expresses at least five different spliced messages (44, 45) and the largest of these, the 13s message, encodes a 289 amino acid product that is able to transactivate and transrepress (46, 47). We therefore inserted a cDNA of this message into the pAc373 baculovirus transfer vector. Expression of E1A protein following infection of Sf9 insect cells by the recombinant virus containing the E1A 13s cDNA sequences was monitored by immunoblotting using a polyclonal serum raised against bacterially synthesised E1A protein (R1) as well as a monoclonal antibody (M2) that

specifically recognises the 13s-encoded product. Time course experiments revealed that although E1A protein continued to be synthesised up to 48 hrs after infection, extensive degradation was evident beginning 28–30 hrs post-infection (data not shown). For purification of the protein, therefore, we harvested the infected cells 28 hr after infection when the majority of the protein was intact. As shown in Fig. 1, the predominant protein species detected by the antibody, migrated with an apparent molecular weight of 47 kd. This species comigrated with a polypeptide detected in extracts from human 293 cells which contain and express the adenovirus E1A gene (39). Significantly however, this species has a higher apparent molecular weight than the E1A polypeptide synthesised in *E. coli*, which migrates at approximately 45 kd (21, 25, 48). A number of reports have shown that the faster migration of bacterially produced E1A protein is due to its lack of phosphorylation and indeed upon incubation of this protein with an appropriate mammalian cell extract, such as a rabbit reticulocyte extract, phosphorylation of the protein can take place resulting in a shift in its electrophoretic mobility such that it comigrates with the phosphorylated protein detected in infected cells (48). Presumably therefore, phosphorylation of E1A results in a conformational change that affects its mobility. The observation that most of the E1A protein synthesised in the infected insect cells migrates with an apparent molecular weight of 47 kd rather than 45 kd, strongly suggests that the protein is efficiently phosphorylated.

To investigate the extent of modification in more detail, 35 S-methionine labelled proteins expressed in infected Sf9 cells were immunoprecipitated with the monoclonal antibody M2, and resolved by two dimensional PAGE. The polypeptides were

Figure 3. *In vitro* activation of polymerase II and III transcription by baculovirus produced E1A protein. **A.** Transcription from the polII transcribed E2A and TK promoters was analysed in whole cell extracts in the absence or presence of purified E1A protein. The transcripts were assayed by primer extension (Materials and Methods). The arrows designate the expected extended products from transcripts initiated from the previously characterised *in vivo* transcription initiation sites; the upper arrow designates the E2A major start site and the lower arrow the TK major start site. **B.** Transcription of the polIII transcribed adenovirus VA1 gene was measured in whole cell HeLa extracts as described in Materials and Methods. In lane 1, no E1A protein was added to the extract. In lanes 2 (50 pgms), 3 (500 pgms), 4 (5 ngms) and 5 (50 ngms) the extract was pre-incubated with increasing amounts of purified E1A protein.

compared to those immunoprecipitated from HeLa cells infected with the adenovirus mutant pm975 (49) which expresses the larger 289 amino acid E1A polypeptide only. As shown in Figure 2, the insect cell produced E1A comprises of multiple species and the pattern was very similar to that seen with E1A protein expressed in pm975 infected HeLa cells. Since the complexity of species revealed by two-dimensional PAGE is almost entirely due to differential phosphorylation of the E1A protein, the result suggests that modification of the protein in insect and mammalian cells is similar.

Immunoaffinity purification of E1A protein expressed in infected insect cells

E1A protein expressed in baculovirus infected insect cells was purified by immunoaffinity chromatography using a modification of the method of Simanis and Lane (50) (see Materials and Methods). Eluted fractions were screened for the presence of E1A protein by immunoblotting and the peak fractions electrophoresed through SDS-PAGE and stained with Coomassie Blue. The purified protein obtained by this method was consistently greater than 80% pure.

We estimate that 10–25 mg of E1A protein was synthesised

per 10^9 infected Sf9 insect cells. Thus the extent of synthesis was very much lower than that obtained with polyhedrin or a number of recombinant products produced by this method. The relatively low level is probably due to two factors: First the infected cells had to be harvested early due to the problems with degradation of the protein at later infection times. Second, the E1A protein in mammalian cells has a very short half-life (approximately 20–30 minutes) (51–53); although we have not directly measured the half life in insect cells, it is probably also short and hence limits the extent of E1A accumulation.

The E1A protein activates transcription *in vivo* and *in vitro*

The purified E1A protein produced in the insect cells was biologically active when tested in microinjected *Xenopus laevis* oocytes. Transcription from an E3CAT reporter gene was stimulated more than 20-fold by co-injection of the purified protein (data not shown). Similar results have previously been obtained with E1A protein overexpressed and purified from bacteria (21).

Attempts by us to stimulate transcription *in vitro* by bacterially synthesised protein had not been successful although some stimulatory activity by bacterially produced protein has been

Figure 4. Characterisation of E1A stimulated VA1 transcription *in vitro*. **A.** *In vitro* transcription of VA1 was assayed as described in Materials and Methods. In lane 1, no E1A was added to the extract. In lanes 2–5, the extract was pre-incubated for 30 minutes with 100 ng of E1A protein prior to the addition of template DNA and nucleotide triphosphates. In lane 3, rabbit polyclonal antibody against E1A protein was also added to the extract at the time of E1A addition. Similarly, in lane 4, M73 monoclonal antibody was added to the extract and in lane 5 a purified non-specific preparation of rabbit IgG. **B.** Lanes 1 and 2 show VA1 transcription in the absence and presence of 100 ng E1A protein respectively. Lanes 3 and 4 additionally contained anti-E1A polyclonal Ab. For lane 3, the Ab was added to the extract at the time of E1A addition and prior to addition of DNA template and triphosphates. For lane 4 however, the Ab was added 45 minutes after E1A addition and 15 minutes after DNA template addition. In all cases, triphosphates were added last and the transcription reaction allowed to proceed for 60 minutes.

described by Spangler *et al.* (19). This failure was the major impetus for expressing the protein in the baculovirus system. We therefore investigated whether the insect derived immunopurified protein was capable of stimulating transcription *in vitro* from two adenoviral promoters, the E2A and MLP promoters. Both these promoters are sensitive to E1A activation *in vivo* although the degree of stimulation of the MLP by E1A is modest compared to that seen with the E2A and other early promoters. Whole cell extracts prepared from HeLa cells were incubated in the absence or presence of purified E1A protein for 30 minutes. Template DNA was subsequently added followed 30 minutes later by NTPs to begin the transcription reaction. As shown in Fig. 3A, E1A protein resulted in a modest stimulation of transcription from the E2A promoter. The extent of stimulation varied considerably from one cell extract to the next, the range being a stimulation

of 5–10 fold. The reasons for the variation are not clear. Similar results were obtained with the MLP promoter (data not shown). In contrast we never detected an increase in expression from the herpes simplex thymidine kinase gene promoter which *in vivo* is relatively insensitive to E1A action (54).

A far more significant stimulation was detected when we investigated the effect of the purified E1A protein on transcription of the VA1 gene of adenovirus, an RNA polymerase III transcribed gene (Fig. 3B). It had previously been shown that this gene was stimulated *in vivo* by the 289 amino acid product of E1A (9–11). Pretreatment of whole cell extracts with increasing amounts of E1A protein resulted in significant increases in VA1 transcription. From a number of different experiments, the average stimulation was found to be approximately 50-fold when 100 ng E1A protein was incubated

with 150 μ g of whole cell extract (see Figs. 3B, 4A, 4B). The degree of stimulation was sensitive to the ratio of extract to DNA template; increasing the concentration of template relative to extract resulted in an increase in transcription in the absence of E1A and a decrease in the degree of stimulation although some stimulation was always seen (data not shown). To confirm that the stimulation was due to E1A protein and not a contaminating activity, we preincubated the protein with either E1A specific polyclonal or monoclonal antibody prior to the addition of extract and DNA template. As shown in Fig. 4A, such treatment abolished the stimulation (lanes 3, 4). In contrast, preincubation with non-specific rabbit IgG had no effect (Fig. 4A, lane 5).

In order to obtain full inhibition, it was necessary to add the antibody before the addition of extract and template DNA. As shown in Fig. 4B, preincubation of extract and E1A protein for 30 minutes, followed by the addition of E1A specific antibody and DNA template resulted in elevated levels of transcription (compare lanes 3, 4).

DISCUSSION

We have successfully overproduced the product of the adenovirus E1A 13s message using the helper-independent baculovirus expression system. The resulting product is phosphorylated and although the analysis is not sufficiently rigorous to conclude that every site that is phosphorylated in mammalian cells is also phosphorylated in infected Sf9 insect cells, the one- and two-dimensional gel electrophoresis analysis would strongly suggest that the phosphorylation pattern is very similar. The protein should therefore prove to be very useful for studying E1A activity *in vitro*.

The insect produced E1A protein can stimulate both RNA polymerase II and polymerase III transcription *in vitro*. The degree of polII stimulation using either the E2A or the major late promoter as template was modest (5–10 fold) but was similar to the degree of stimulation reported by others (19, 20). In contrast, stimulation of the polIII-transcribed VA-gene, was reproducibly large, in some experiments being greater than 50-fold. Although it has been shown previously that extracts prepared from adenovirus infected cells had higher polIII transcription activity than uninfected extracts (9, 11), this is the first report of stimulation of an uninfected extract by the addition of purified E1A protein. The result clearly indicates that E1A-induced stimulation of polIII transcription does not require the synthesis of new protein and must instead depend upon some direct or indirect interaction of E1A with pre-existing transcription factors. The observed stimulatory effect was specifically due to the added E1A protein since monoclonal and polyclonal antibodies to E1A negated the effect. Interestingly however, antibody mediated interference was not observed if the extract was incubated with E1A protein prior to antibody addition. This result may imply that either E1A protein was sequestered in some way and no longer recognised by the antibodies or, perhaps more likely, that E1A modified the extract resulting in an increased capacity for polIII transcription; assuming the modification was relatively stable, subsequent removal of E1A with antibody would have no effect.

The mechanism of E1A transactivation remains unknown. It does not appear to involve sequence specific binding of the protein, and does not require the synthesis of new protein. Models proposed to explain E1A action have therefore revolved around the interaction with or modification of pre-existing transcription

factors. In the case of polIII transcription, E1A appears to mediate its effect via the limiting factor TFIIC. Comparison of adenovirus infected cell extracts versus uninfected extracts has clearly demonstrated that the increased activity of the infected extract is due to an increase in TFIIC activity (10, 11). However, there has been some disagreement concerning the nature of this increase. Using template commitment assays, Yoshinaga *et al.* (11) reported an increase in the abundance of TFIIC in infected extracts, suggesting that stimulation by E1A resulted from an increase in synthesis of this limiting factor. The demonstration in this report that E1A can stimulate *in vitro* would clearly not be compatible with such a model. On the other hand, Hoeffler *et al.* (10) found no difference in TFIIC abundance between infected and uninfected extracts. However, they did find a qualitative difference in TFIIC, an increase in the phosphorylated form of the factor being present in the infected extract. Since they also showed that phosphorylated TFIIC was more efficient in activating transcription *in vitro* than the unphosphorylated form, they proposed that E1A stimulated polIII transcription by stimulating TFIIC phosphorylation. We are currently investigating the phosphorylated state of TFIIC in extracts incubated with the insect synthesised E1A protein. Our results showing that incubation of E1A extract prior to the addition of E1A antibody allows activation to occur would be consistent with such a model.

We have been consistently unsuccessful in stimulating *in vitro* transcription with E1A protein overproduced and purified from bacteria, despite the fact that the same protein is active when microinjected into *Xenopus laevis* oocytes or mammalian cells (21, 40). In contrast, Spangler *et al.* (19), have demonstrated some activation of polIII transcription *in vitro* with bacterially produced protein. It is currently not clear whether our success with the insect produced E1A is due to the fact that it is post-translationally modified or due to its isolation using non-denaturing conditions. However, since E1A protein can be dephosphorylated *in vitro* (25) it should be possible to investigate the role of phosphorylation in E1A transactivation.

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