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Adenovirus stimulation of transcription by RNA polymerase III: evidence for an E1A-dependent increase in transcription factor IIIC concentration

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Human cells expressing adenovirus E1A proteins transcribe transfected tRNA and adenovirus VAI genes at >10-fold higher levels than uninfected HeLa cells. Here we show that the increased transcription observed *in vivo* is reflected in the *in vitro* transcriptional activity of cell extracts. Depletion of E1A protein from these extracts by immunoprecipitation with a monoclonal antibody did not diminish the activity, suggesting that E1A proteins do not stimulate transcription directly. Fractionation of the extracts by chromatography on phosphocellulose suggests that the higher activity of extracts of adenovirus-infected cells was due to increased activity of the transcription factor (TF) which is the limiting component required for specific initiation of tRNA and VAI transcription in extracts of uninfected HeLa cells, i.e. TFIIIC. Template commitment titrations further suggest that the increased TFIIIC activity was due to an increase in the concentration of active TFIIIC. On the basis of these results and recent genetic analyses of early adenovirus promoters, we suggest that E1A proteins stimulate transcription of adenovirus genes indirectly by increasing the effective *in vivo* concentration of the limiting cellular transcription factors required for their transcription.

Key words: RNA polymerase III/adenovirus-infected cells/transcription factor IIIC/E1A proteins

Introduction

Adenovirus E1A and herpes virus immediate early proteins have attracted attention because of their ability to stimulate transcription of early viral genes (Jones and Shenk, 1979b; Berk *et al.*, 1979; Nevins, 1981; Kit *et al.*, 1978; Preston, 1979; Watson and Clements, 1980). Several lines of evidence suggest that these proteins do not act by interacting with specific DNA sequences near the sites of transcription initiation. First, although herpes and adenoviruses share little sequence homology, herpes virus immediate early proteins (IE proteins) stimulate transcription from early adenovirus promoters (Feldman *et al.*, 1982). Similarly, adenovirus E1A protein stimulates transcription of an early herpes virus gene (Everett and Dunlop, 1984). Second, the ability of these proteins to stimulate transcription is not limited to viral genes. The E1A and IE proteins stimulate transcription of a number of mammalian genes in transient transfection assays (Green *et al.*, 1983; Gaynor *et al.*, 1984; Svensson and Akusjarvi, 1984; Allan *et al.*, 1984). Third, extensive genetic analyses of the adenovirus early region 2A (Elkaim *et al.*, 1983; Kingston *et al.*, 1984; Imperiale *et al.*, 1985; Murthy *et al.*, 1985) and E3 (Leff *et al.*, 1985) promoters have not revealed sites required for stimulation of transcription by E1A proteins which can be distinguished from promoter sequences. The wild-type promoter and partially defective mutants of it are equally stimulated by E1A proteins. Hence, no site at which E1A proteins might act has been uncovered by these extensive mutagenesis studies. Finally, we find that E1A proteins extracted from adenovirus-infected HeLa cells do not have DNA-binding activity (K.Spindler, A.Tsukamoto and A.J.Berk, unpublished observations). E1A protein expressed in *Escherichia coli* also fails to display DNA-binding activity (Ferguson *et al.*, 1985). These results suggest that the E1A and IE proteins have similar activities which indirectly stimulate transcription of a variety of genes when they are newly introduced into cells, either by infection of a virus or by calcium phosphate-mediated DNA transfection.

In contrast to the ability of E1A protein to stimulate transcription of a variety of transfected genes, endogenous genes are subject to much more selective activation. Although the transcription of transfected preproinsulin and β-globin genes is stimulated by E1A protein (Gaynor *et al.*, 1984; Green *et al.*, 1983), the endogenous preproinsulin and β-globin genes are not activated. Moreover, transcription of the vast majority of endogenous genes is not increased in adenovirus-infected cells (Beltz and Flint, 1979; Babich *et al.*, 1983). On the other hand, transcription of a small number of specific endogenous genes, including the *hsp-70* heat shock gene (Kao and Nevins, 1983) and a β-tubulin gene (Stein and Ziff, 1984) is stimulated by E1A protein after adenovirus infection.

A further testament to the generality of transcriptional activation by E1A and IE proteins is that the process is not limited to genes transcribed by RNA polymerase II. Transfected class III genes (genes transcribed by RNA polymerase III) are expressed at >20-fold higher levels in cells expressing E1A (Berger and Folk, 1985; Gaynor *et al.*, 1985; Hoeffler and Roeder, 1985) or IE proteins (Gaynor *et al.*, 1985) than in uninfected HeLa cells. Thus, transcription of transfected class III genes as well as class II genes is stimulated by these viral *trans*-acting proteins. Similar mechanisms may operate in the activation of both classes of genes, since for both classes of genes the effect is largely restricted to newly introduced genes (Gaynor *et al.*, 1985).

Specific initiation of class III genes *in vitro* is readily detected using HeLa cell extracts (Wu, 1978; Weil *et al.*, 1979). If the increase in class III gene transcription observed *in vivo* were reflected *in vitro* using soluble extracts of adenovirus-infected HeLa cells, fractionation of the extracts might reveal which component(s) is responsible for the transcriptional stimulation. Fractionation of S100 extracts prepared from uninfected HeLa cells (Segall *et al.*, 1980; Fuhrman *et al.*, 1984; Lassar *et al.*, 1983) has determined that at least two components, in addition to RNA polymerase III (polIII), are essential for transcription of tRNA and adenovirus VA genes *in vitro*. These transcription factors have been referred to as TFIIIC and TFIIIB (Segall *et al.*, 1980; Lassar *et al.*, 1983). They have been partially purified and each

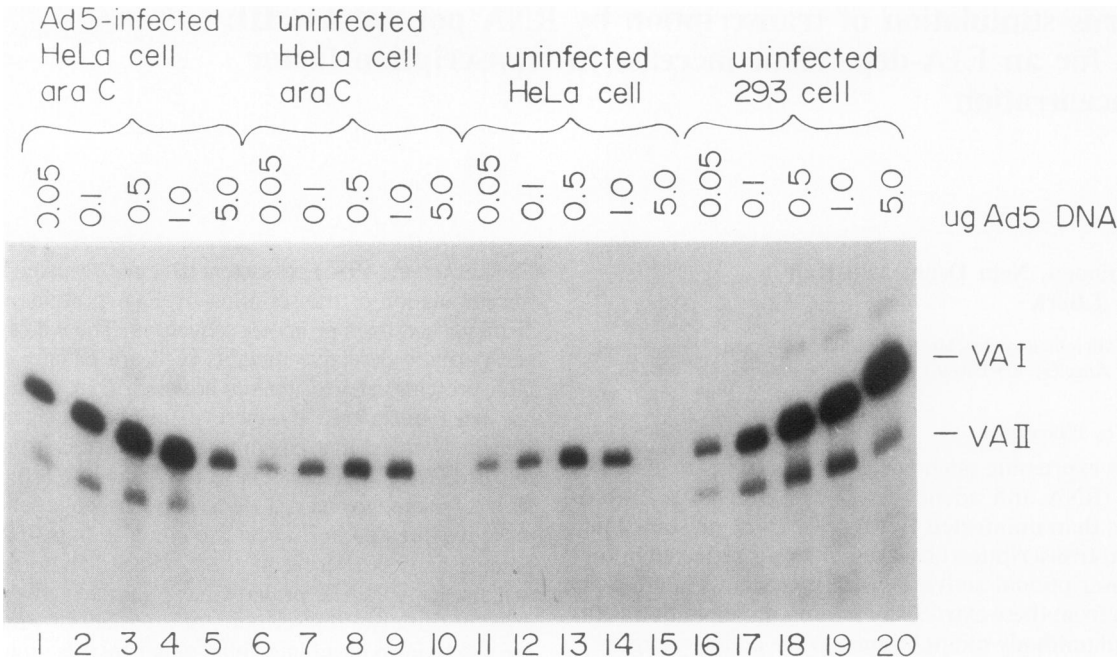

Fig. 1. Relative activities of S100 extracts. The figure shows an autoradiograph of labeled VAI RNA products products from *in vitro* transcription reactions. S100 extracts were made from Ad5-infected **(lanes 1–5)** or mock-infected **(lanes 6–10)** ara-C-treated HeLa cells 36 h post-infection. Mock-infected HeLa cells, without ara-C treatment, were used to prepare S100 extracts 16 h post-mock infection **(lanes 11–15)**. S100 extracts were also prepared from exponentially growing 293 cells **(lanes 16–20)**. 20 μg of S100 extract protein were used in each transcription reaction with 0.05 **(lanes 1,6,11,16)**, 0.1 **(lanes 2,7,12,17)**, 0.5 **(lanes 3,8,13,18)**, 1.0 **(lanes 4,9,14,19)** and 5.0 μg **(lanes 5,10,15,20)** purified Ad5 DNA as template. Reaction conditions were as described in Materials and methods.

appears to be a single protein (Lassar *et al.*, 1983; Fuhrman *et al.*, 1984), but their purification to homogeneity has not yet been achieved. Partially purified TFIIIC has sequence-specific DNA binding activity as demonstrated by protection of the internal promoter regions of tRNA and adenovirus VAI genes from nuclease digestion (Fuhrman *et al.*, 1984; Lassar *et al.*, 1983). TFIIIB binds to TFIIIC-DNA complexes to form a 'stable transcription complex' composed of a tRNA or VAI gene, TFIIIC and TFIIIB (Lassar *et al.*, 1983). These stable transcription complexes are the substrate for multiple rounds of accurate transcription by polIII. TFIIIC is the limiting component required for tRNA or VAI RNA transcription in S100 extracts of uninfected HeLa cells (Fuhrman *et al.*, 1984). Since TFIIIC has not yet been purified to homogeneity, it is possible that the previously described TFIIIC activity is due to more than one protein. For the purpose of this paper, we apply the term TFIIIC to the limiting component required for specific initiation of tRNA and VAI transcription in S100 extracts of uninfected HeLa cells.

Here we report that S100 extracts of wild-type adenovirus-infected HeLa cells have increased activity for the *in vitro* transcription of VAI and tRNA genes compared with extracts of uninfected or E1A mutant-infected HeLa cells. Analysis of these extracts suggests a mechanism for transcription stimulation *in vivo* and for the preferential activation of transfected compared with endogenous class III genes.

Results

To determine whether the stimulatory effect of E1A proteins on the transcription of class III genes observed *in vivo* (Berger and Folk, 1985; Gaynor *et al.*, 1985; Hoeffler and Roeder, 1985) could be detected and analyzed *in vitro*, we prepared S100 extracts from uninfected and adenovirus-infected HeLa cells and from the adenovirus-transformed 293 cell line. This cell line expresses Ad5 E1A and E1B proteins constitutively (Graham *et al.*, 1977; Lassam *et al.*, 1979). Transcription of adenovirus VAI RNA *in vitro* was analyzed in reactions containing various amounts of purified Ad5 DNA and a constant amount (20 μg) of S100 extract protein (Figure 1). The 293 cell S100 extract was more active than extracts of uninfected HeLa cells at all DNA concentrations tested. The optimum Ad5 DNA template concentration for the uninfected HeLa cell S100 extract was between 12.5 and 25 μg/ml, results consistent with previous findings (Weil *et al.*, 1979). When the template concentration was raised considerably, inhibition of VAI transcription occurred. The 293 cell extract was less inhibited at high DNA concentrations. At the optimum Ad5 DNA template concentration for transcriptions by the uninfected HeLa cell extract, the 293 cell extract transcribed ~10 times more VAI RNA. The 293 cell extract was also 10 times more active in the transcription of a cloned $tRNA^{Arg}$ gene (Silverman *et al.*, 1979; data not shown).

In vivo expression of transfected tRNA genes was much greater in HeLa cells treated with cytosine arabinoside (ara-C) and infected with wild-type adenovirus compared with ara-C-treated HeLa cells which were mock-infected or infected with the E1A deletion mutant dl312 (Berger and Folk, 1985; Gaynor *et al.*, 1985). In these transient transfection assays, ara-C was added to inhibit viral DNA replication and entry into the late stage of infection, preventing cell death during the course of the transfection assay. To determine if this increase in class III gene expression observed *in vivo* in Ad5-infected, ara-C-treated HeLa cells could also be observed in the *in vitro* activity of extracts, we prepared S100 extracts of these cells at various times post-infection. Control extracts were also prepared, in parallel, from HeLa cells which were not treated with ara-C, mock-infected cells treated with ara-C and ara-C-treated cells infected with

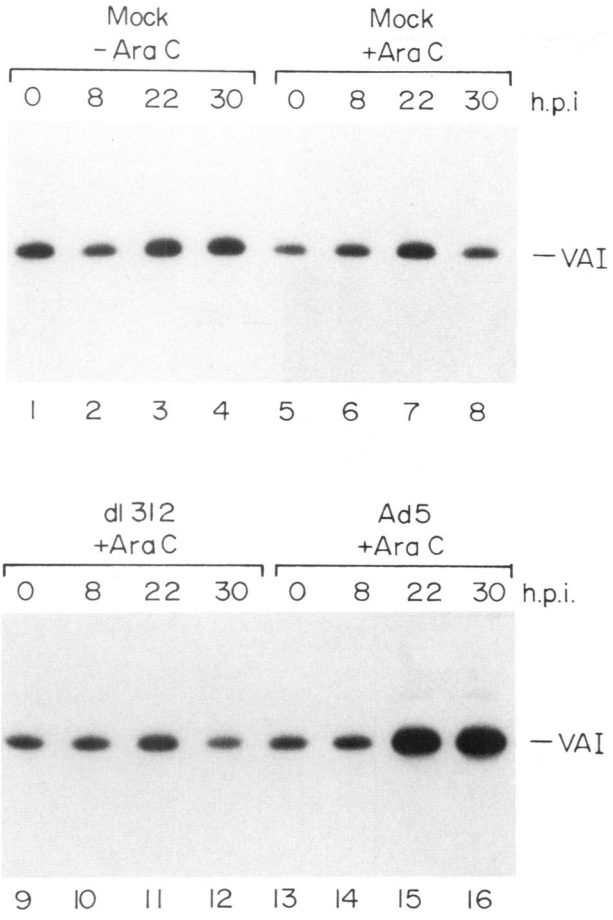

Fig. 2. *In vitro* RNA transcription with extracts prepared at various times after infection of ara-C-treated HeLa cells. An autoradiograph of labeled RNA products from transcription reactions using 30 μg S100 protein prepared from mock-infected HeLa cells **(lanes 1–4)**, mock-infected HeLa cells treated with ara-C **(lanes 5–8)**, dl312-infected HeLa cells treated with ara-C **(lanes 9–12)** and Ad5-infected HeLa cells treated with ara-C **(lanes 13–16)**. Extracts were made 0 h **(lanes 1,5,9,13)**, 8 h **(lanes 2,6,10,14)**, 22 h **(lanes 3,7,11,15)** and 30 h **(lanes 4,8,12,16)** post-infection. 0.75 μg Ad5 DNA was used as template in the transcription reactions.

dl312. There were only small differences in the transcriptional activities of these control extracts prepared at various times after infection (Figure 2). Treatment of cells with ara-C alone did not result in an increase in transcriptional activity. On the other hand, extracts prepared from Ad5-infected HeLa cells treated with ara-C showed increased VAI transcriptional activity when made at 22 and 30 h post-infection. Infection with dl312 did not produce the same increase in transcriptional activity of S100 extracts, indicating that an E1A function is required to observe the increased class III gene transcriptional activity of S100 extracts just as it is for increased transcription *in vivo* in transfection experiments. Figure 1 shows the activity of extracts from a separate experiment assayed at varying DNA template concentrations. Extracts of Ad5-infected, ara-C-treated HeLa cells were 5 – 10 times more active than control extracts through a range of DNA concentrations.

To compare accurately the rate of VAI RNA synthesis in extracts from Ad5- and dl312-infected HeLa cells, a time course of the *in vitro* transcription reaction was examined. At various times after initiating the reaction in the presence of [α-^{32}P]GTP, aliquots were removed and the reaction stopped. Following an initial lag, extracts from Ad5-infected HeLa cells exhibited an ~7-fold increase in the linear rate of RNA synthesis as well as a decrease in the initial lag period compared with extracts from dl312-infected HeLa cells (Figure 3).

Optima for KCl and $MgCl_2$ concentrations in the transcription reactions were identical for extracts prepared from wild-type infected ara-C-treated HeLa cells and uninfected ara-C-treated HeLa cells (data not shown), and were identical to those reported previously for extracts of untreated HeLa cells (Weil *et al.*, 1979). In the experiments described below we utilized extracts from infected or uninfected HeLa cells treated with ara-C. Results of experiments in which HeLa cells were not treated with ara-C are presented at the end of the Results.

Effects of exogenously added RNA polymerase III

To determine whether the increased transcriptional activity observed in extracts of wild-type infected cells was due to increased amounts of RNA polIII, we added purified polIII to the transcription reactions. Using an RNA polIII activity assay (Roeder, 1974) we found that 20 μg of S100 extract protein from mock-infected cells contained approximately one unit of polIII activity, while two units of polIII activity were measured in an Ad2-infected HeLa cell S100 extract. Supplementation of the S100 extracts with exogenous polIII did not result in increased transcription of VAI RNA in *in vitro* reactions (Figure 4). These results are consistent with those of Weil *et al.* (1979) and indicated that polIII is not the limiting component for VAI transcription in these S100 extracts.

Depletion of E1A proteins from S100 extracts of 293 and Ad5-infected HeLa cells

Next we considered the possibility that extracts derived from either 293 cells (which constitutively express the E1A proteins) or Ad5-infected HeLa cells have increased transcriptional activity compared with extracts of mock- or dl312-infected HeLa cells because of the direct action of E1A proteins in the active extracts. To determine if this was the case, extracts of 293 cells and Ad5- infected HeLa cells were depleted of E1A protein by immunoprecipitation using anti-E1A monoclonal antibodies (αE1A R7, A.Tsukamoto and A.Berk, unpublished results) covalently coupled to Sepharose. As controls, extracts were treated with an unrelated monoclonal antibody (α-ICP4) bound to Sepharose, or carried through the procedure without the addition of immunoaffinity resin. E1A proteins present in the supernatant and pellet fractions after immunoprecipitation of extracts from infected HeLa cells were analyzed by Western immunoblotting (Figure 5A). Treatment of the extract with the anti-E1A monoclonal resin removed detectable E1A protein from the supernatant fraction (lane 4), leaving it associated with the pellet (lane 5). Treatment with the control anti-ICP4 antibody left most of the E1A protein in the supernatant fraction (lane 2). A fraction of the E1A protein aggregated during storage of the S100 extract and sedimented non-specifically into the anti-ICP4 pellet fraction (lane 3). The control and immunoprecipitated supernatant fractions were used to transcribe the VAI gene *in vitro*. Although treatment with the anti-E1A monoclonal antibody resin removed most of the E1A protein from the extract (Figure 5A), the transcriptional activity of the extract was undiminished (Figure 5B). Extracts of 293 cells were similarly depleted of E1A protein by immunoprecipitation with anti-E1A monoclonal antibody. Again no significant decrease in the activity of these E1A-depleted extracts was apparent (data not shown). The simplest interpretation of these results is that E1A protein does not stimulate VAI transcription directly, but rather induces or activates other fac-

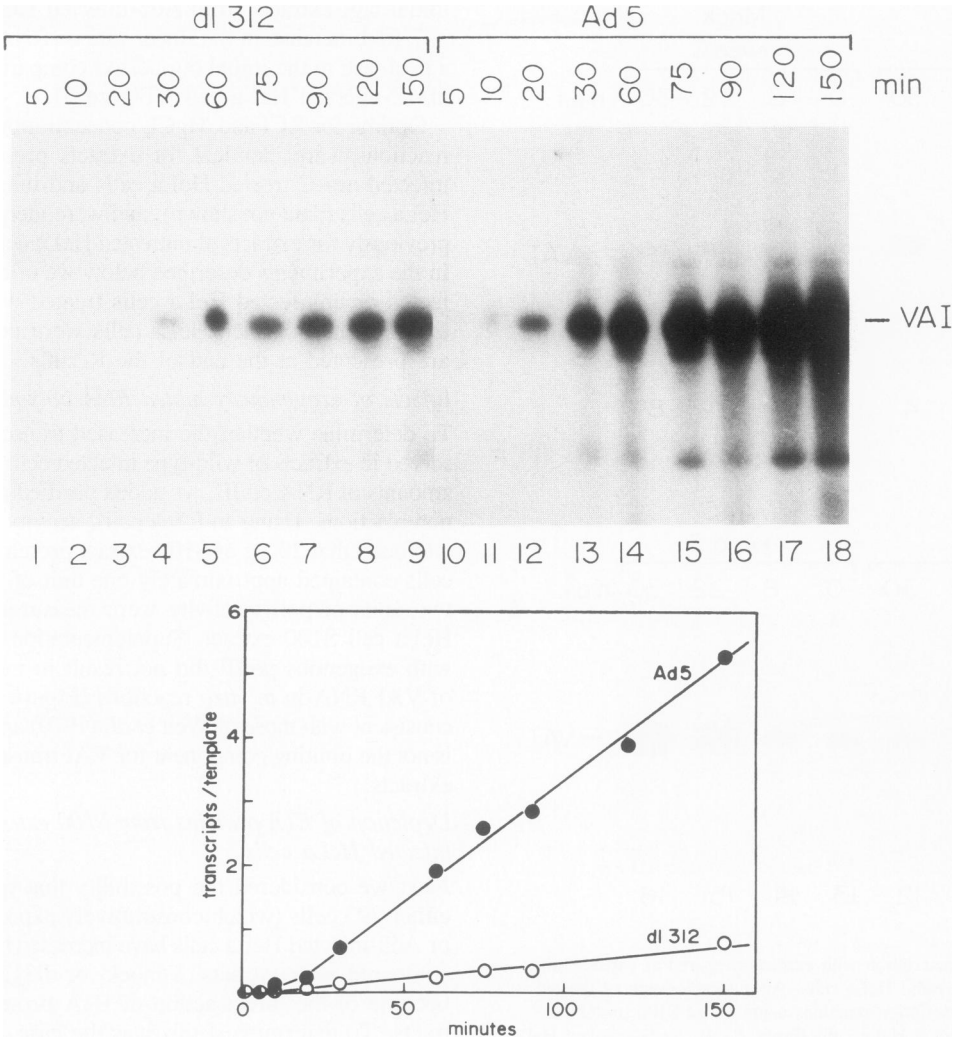

Fig. 3. Kinetics of *in vitro* VAI transcription using extracts of Ad5- and dl312-infected, ara-C-treated HeLa cells. The upper panel shows an autoradiograph of labeled VAI RNA products from *in vitro* transcription reactions incubated for increasing periods of time. The labeled VAI RNA was excised from the dried gel and Cerenkov c.p.m. were determined. The number of transcripts synthesized per template was calculated from the specific activity of the [α-^{32}P]GTP and the number of G residues in the VAI RNA product. Results for Ad5- (●) and dl312- (○) infected HeLa cell extracts are plotted in the lower panel. 20 μg S100 extract protein and 0.5 μg pVA template were used in the reactions.

tors which result in the increased activity of S100 extracts. However, these experiments alone cannot rule out the possibility that small amounts of E1A protein remaining after immunoprecipitation may be sufficient to stimulate transcription. On the other hand, fractionation studies (see below) also indicated that E1A protein does not stimulate VAI transcription directly.

Fractionation of S100 extracts

Fractionation of mock-infected and wild-type adenovirus-infected HeLa cell S100 extracts by phosphocellulose chromatography was conducted, in parallel, according to the procedure of Segall *et al.* (1980). As they demonstrated with S100 extracts of uninfected HeLa cells, fractions B, C and polIII reconstituted specific transcription of VAI RNA (Figure 6A, lanes 1 and 2). Transcription of VAI RNA in a reaction reconstituted with fractions B and C prepared from Ad2-infected HeLa cells was five times greater than reactions reconstituted with B and C fractions prepared from mock-infected cells. Addition of fraction A and/or fraction D prepared from Ad2-infected cells did not further stimulate activity. These results indicate that the component(s) responsible for the increased transcriptional activity of extracts of wild-type infected cells was in the B and/or C fractions.

To determine whether one or both of the fractions were responsible for the increased activity of Ad2-infected cell S100 extracts, we measured the activity of these fractions separately. To assay TFIIIB activity in B fractions prepared from Ad2 and mock-infected cells, decreasing amounts of B fraction protein were added to constant amounts of polIII, DNA template and fraction C from uninfected HeLa cell extracts and *in vitro* transcription reactions were performed (Figure 7, upper panel). Fraction B from Ad2- and mock-infected HeLa cells showed similar activities. These results indicate that fraction B from Ad2-infected HeLa cell extracts did not contain the component(s) which gave rise to the increased transcriptional activity observed in the unfractionated Ad2-infected HeLa cell extract.

To assay transcription factors in fraction C, a constant amount of polIII, DNA template and fraction B from uninfected cells was added to decreasing amounts of fraction C prepared from infected or uninfected cells (Figure 7, lower panel). The level of VAI synthesized at each of the indicated amounts of fraction C protein was greater with fraction C prepared from infected compared with uninfected cells. Increasing VAI RNA was synthe-

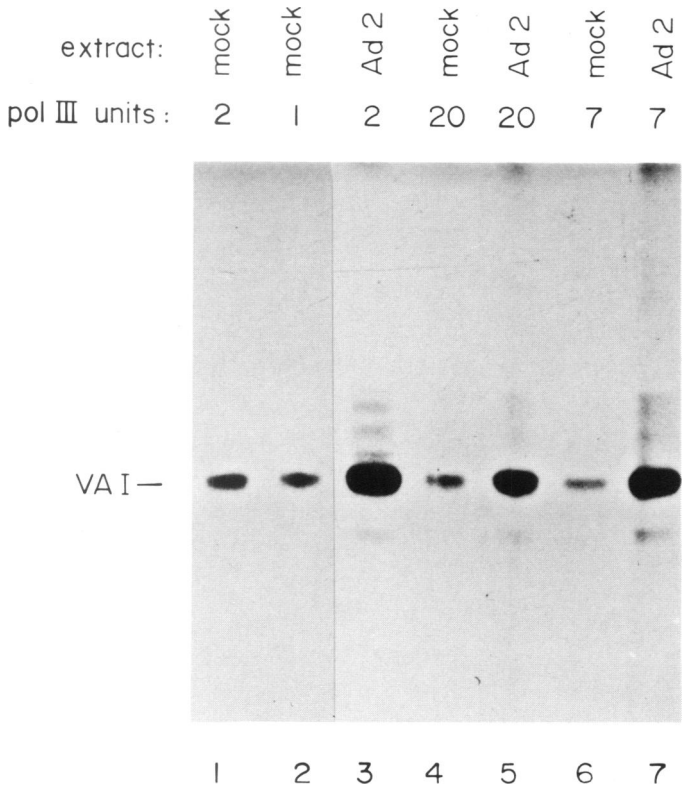

Fig. 4. Addition of exogenous RNA polymerase III to *in vitro* transcription reactions. Ad2-infected HeLa cell S100 extracts contained 2 units of endogenous polIII activity **(lane 3)**, while mock-infected cell extracts contained 1 unit **(lane 2)** per 20 μg protein. RNA polymerase III (purified as described in Materials and methods), was added to *in vitro* reactions with mock-infected extract to a total of 2 units **(lane 1)**, 7 units **(lane 6)** and 20 units **(lane 4)** of polIII activity, and to reactions with the Ad2-infected cell extract to a total polIII activity of 7 units **(lane 7)** and 20 units **(lane 5)** per reaction. 20 μg of S100 extract protein and 1 μg purified Ad5 template were used in the reactions.

sized in response to increasing fraction C protein, except for the increase from 2.5 μg to 5 μg of fraction C from infected cells. This would be the expected result if TFIIIC was in excess over TFIIIB when 5 μg of fraction C from infected cells was added to the reaction. At 1.25 and 2.5 μg of fraction C protein, five times more VAI RNA was synthesized with fraction C prepared from infected cells compared with uninfected cells. Since the S100 extracts from which these fractions were prepared also showed a 5-fold greater activity for the infected extract, the difference in the activities of the unfractionated extracts could be accounted for by the increased activity of fraction C from infected cells.

E1A protein in the phosphocellulose fractions prepared from Ad2-infected cells was analyzed by Western immunoblotting using an antibody to an E1A fusion protein produced in *E. coli* (Figure 6B). Most of the E1A protein flowed through the phosphocellulose column and was detected in fraction A. A small amount was detected in fraction B and still less in fraction C. Since the increased transcriptional activity of infected cell extracts was due to activities in the C fraction, and addition of fraction A did not further stimulate transcription, the results of the fractionation analysis agree with the immunoprecipitation experiment. Both indicate that E1A protein does not act directly to stimulate class III gene transcription *in vitro*. Furthermore, addition of fraction A from infected cells to fraction B and C from uninfected cells did not stimulate *in vitro* VAI transcription (data not shown).

Evidence for increased TFIIIC in extracts of Ad5-infected HeLa cells

The results of Figure 7 demonstrate that the increased activity of S100 extracts prepared from adenovirus-infected HeLa cells was due to factors which fractionated in the C fraction on phosphocellulose. This could be due to increased activity of the essential class III transcription factor contained in fraction C, TFIIIC (Segall *et al.*, 1980), or to another factor, potentially encoded or induced by adenovirus, which might stimulate transcription above that observed with TFIIIB and TFIIIC alone. Several observations were more consistent with an increase in TFIIIC activity rather than a distinct stimulatory activity in fraction C. First, when C fractions prepared from uninfected and infected HeLa cells were mixed, the TFIIIC activity measured in the mixture was the average of the two. Similarly, when the unfractionated S100 extracts from infected and uninfected cells were mixed, the mixture had the average *in vitro* transcriptional activity of the two extracts. These are the expected results if adenovirus infection results simply in increased TFIIIC activity. A stimulatory factor, if it acted catalytically or was present in excess, would result in higher than the average activity of these mixtures, and this was not observed.

To test more directly for an activity in the C fraction from infected cells which might stimulate transcription from stable transcription complexes made from TFIIIB and TFIIIC from uninfected cells, we performed the following experiment. Template DNA was pre-incubated with B and C fractions from uninfected cells (UB and UC, respectively) under conditions in which the number of stable transcription complexes which could form was limited by the number of TFIIIB molecules added to the reaction. This condition was met with 2 μg UB protein plus 4 μg UC protein, since addition of more UB protein resulted in proportionally more transcription when nucleotide triphosphates were added (Figure 8, lanes 1–4). The pre-incubation was for 10 min in the absence of nucleotide triphosphates, a time sufficient for stable transcription complexes to form on the VAI gene (Lassar *et al.*, 1983; Fuhrman *et al.*, 1984). Under these conditions, addition of more TFIIIC after the pre-incubation was not expected to stimulate transcription since the number of transcription complexes which could form was limited by the amount of TFIIIB. Accordingly, addition of another 4 μg of fraction UC did not stimulate transcription significantly (lane 5). On the other hand, addition of more TFIIIB to the pre-incubated mixture did stimulate subsequent transcription, as expected (lane 6). The experiment shown in lane 7 tested whether a component in fraction C prepared from infected cells (IC) could stimulate transcription from the fixed number of transcription complexes formed with TFIIIB and TFIIIC from uninfected cells. Little stimulation of VAI transcription was detected after addition of 4 μg of fraction IC protein to the pre-formed transcription complexes (compare lanes 3, 5 and 7). This result demonstrates that fraction IC did not contain an activity which stimulated the rate of transcription from transcription complexes formed with TFIIIB and TFIIIC from uninfected cells. Also, the small quantity of E1A protein detected in fraction C from infected cells (Figure 6B) does not appear to be a minor, modified form of E1A protein which stimulates transcription directly. Rather, the results are consistent with the increased activity of extracts from infected cells resulting from an increase in the activity of transcription factor TFIIIC itself. However, these results do not rule out the possibility of a stimulatory factor in fraction C which must bind to transcription complexes as they assemble. On the other hand, further analyses presented below suggest that an increase in TFIIIC activity in

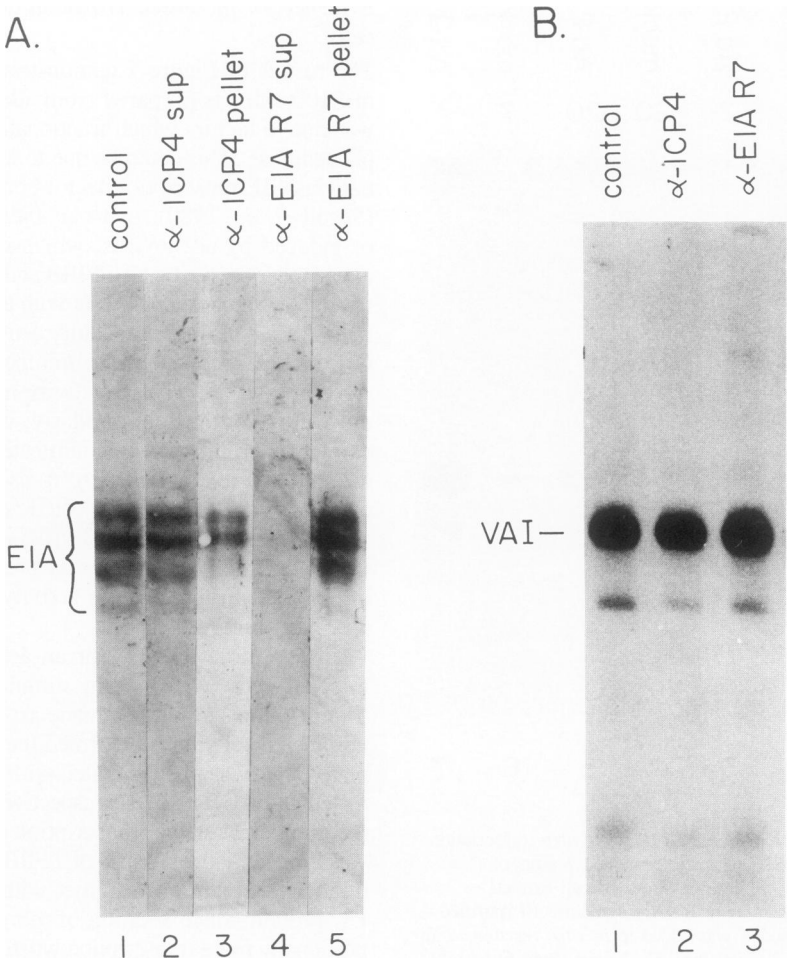

Fig. 5. *In vitro* transcriptional activity of E1A protein-depleted extract. S100 extracts were prepared from Ad5-infected ara-C-treated HeLa cells 36 h post-infection. E1A protein was removed from the extracts by immunoprecipitation with anti-E1A monoclonal antibody R7 coupled to Sepharose as described in Materials and methods. As controls, extracts were similarly treated with a monoclonal antibody to the herpes simplex virus ICP4 protein, or with Sepharose alone. **(A)** 20 μg of untreated extract protein **(lane 1)**, 20 μg of the supernatant protein following immunoprecipitation with anti-ICP4 **(lane 2)**, or anti-E1A R7 **(lane 4)**, and protein eluted from the anti-ICP4 pellet **(lane 3)** and anti-E1A R7 pellet **(lane 5)** equivalent to the immunoprecipitate from 20 μg of extract protein, were analyzed by Western immunoblotting using a polyclonal anti-E1A protein rabbit serum (Spindler *et al.*, 1984). **(B)** Products of 60 min *in vitro* transcription reactions using 20 μg of supernatant protein following immunoprecipitation with Sepharose alone **(lane 1)**, with anti-ICP4 Sepharose **(lane 2)** and with anti-E1A R7 Sepharose **(lane 3)**.

extracts of infected cells results from a simple increase in the concentration of active TFIIIC molecules.

As another test for TFIIIC activity in extracts of Ad5- and dl312-infected HeLa cells, we performed template commitment assays using the unfractionated extract, as described by Fuhrman *et al.* (1984). The template commitment assay is based on the ability of the first template (DNA I), added in a pre-incubation with an S100 extract in the absence of nucleoside triphosphates, to sequester transcription factors and thus pre-empt the transcription of a second template (DNA II) added subsequently. The preferential transcription of the first template reflects a stable interaction formed between the first template and some titratable limiting factor in the extract. Fuhrman *et al.* (1984) showed that in S100 extracts prepared from uninfected HeLa cells, this limiting component is contained in phosphocellulose fraction C. They did this by showing that the inhibition of DNA II transcription caused by pre-incubation of the extract with DNA I can be reversed by addition of fraction C. This is because the transcription factor in fraction C (TFIIIC) is the limiting transcription factor in the S100 extract of uninfected cells. When all TFIIIC molecules are sequestered on DNA I, none are available for binding to DNA II. However, if additional TFIIIC is added by addition of fraction C, it interacts with DNA II and excess TFIIIB in the unfractionated S100 extract, resulting in transcription of DNA II. Fraction C was said to 'rescue' transcription of the second template.

As shown by Fuhrman *et al.* (1984) for S100 extracts of uninfected HeLa cells, we found that using extracts of Ad5-infected, ara-C-treated HeLa cells, fraction C, but not fraction B rescued transcription of the second template (data not shown). Therefore in S100 extracts of both Ad5-infected and uninfected HeLa cells a component of fraction C (TFIIIC) is the limiting component required for the formation of a stable transcription complex. This allowed us to use the template commitment assay to compare the concentrations of TFIIIC in Ad5- and dl312-infected HeLa cell extracts. This was done by determining the amount of the first DNA added to a pre-incubation with an S100 extract which was required to completely sequester TFIIIC and prevent the transcription of a second template added subsequently.

A comparative titration of this type for Ad5- and dl312-infected cell extracts is shown in Figure 9. The titration end point, as judged by the quantity of DNA I that must be added to the

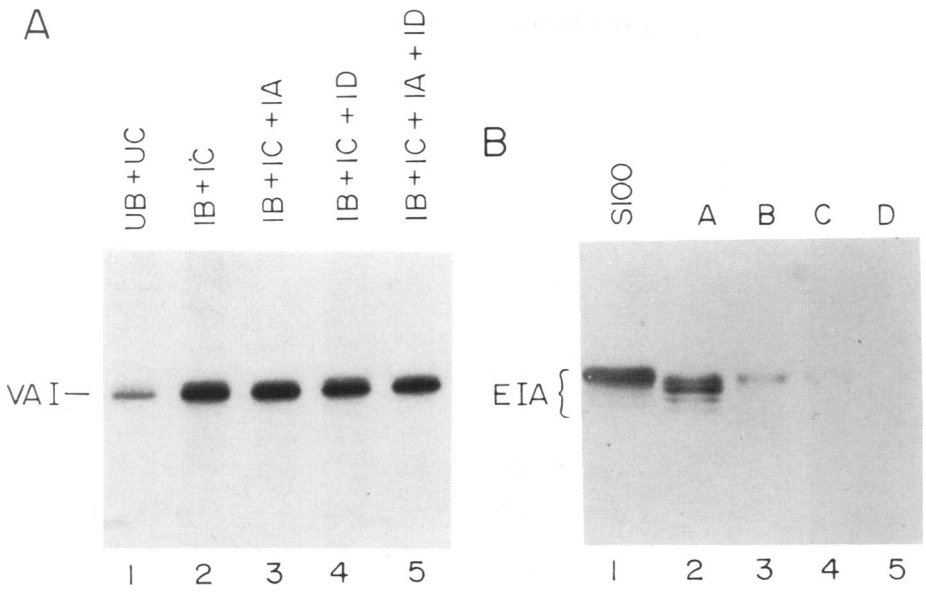

Fig. 6. (A) *In vitro* transcription using phosphocellulose fractions from Ad2-infected or uninfected HeLa cells. S100 extracts were prepared in parallel from mock-infected and Ad2-infected, ara-C-treated HeLa cells at 30 h post-infection. Fractionation of the extracts by chromatography on phosphocellulose was performed as described (Segall *et al.*, 1980). The S100 extract protein fractionated such that 26 parts were in fraction A, to 10 parts in fraction B, to four parts in fraction C, to one part in fraction D. Each transcription reaction contained the indicated fractions plus 10 units polIII and 1 μg Ad5 DNA template. UB and UC designate fraction B and fraction C isolated from uninfected cells, respectively. IA, IB, IC and ID designate fractions A, B, C and D from Ad2-infected cells. *In vitro* reactions contained 26 μg fraction A protein, 10 μg fraction B protein, 4 μg fraction C protein and 1 μg fraction D protein as indicated. **(B)** Western immunoblot analysis of E1A proteins in phosphocellulose fractions. Unfractionated S100 extract and phosphocellulose fractions from Ad2-infected HeLa cells were boiled in Laemmli gel sample buffer and resolved by electrophoresis on a SDS-polyacrylamide slab gel. 80 μg S100 extract, 52 μg fraction A, 20 μg fraction B, 8 μg fraction C and 2 μg fraction D were used in the analysis. Western analysis was conducted as described (Spindler *et al.*, 1984) using antiserum to an *E. coli* E1A-*trpE* fusion protein.

pre-incubation to block transcription of DNA II, gives an estimate of the relative concentration of TFIIIC in these two extracts. The pVA clone containing an intact Ad2 VAI gene was used as DNA I which was pre-incubated with the S100 extract. Plasmid pdlVAI which contains a deletion within the VAI gene was used as DNA II, added after the pre-incubation. When no DNA I was added to the pre-incubation, transcription of pdlVAI was evident with both extracts. The gel of the dl312 extract reactions was exposed several times longer than that for the Ad5 extract so that the intensity of the dl VAI band at 0 DNA I was approximately equal for the two autoradiograms. 5 fmol of DNA I almost completely blocked transcription of DNA II by the dl312 extract, while 10 fmol prevented DNA II transcription. In contrast, 40 – 80 fmol of DNA I were required to block DNA II transcription when the same amount of the S100 extract from Ad5-infected cells was used. These results indicate that there was a 4- to 8-fold greater concentration of the limiting factor required for the formation of stable transcription complexes in the Ad5-infected cell extract compared with the dl312 extract. This limiting transcription factor is referred to as TFIIIC. Since the activity of the unfractionated S100 extract prepared from Ad5-infected HeLa cells was ~5-fold greater than the dl312-infected cell extract in this experiment, the 4- to 8-fold greater TFIIIC concentration in the Ad5 extract could account completely for the increased activity of the extract from Ad5-infected cells.

Class III transcriptional activity in adenovirus-infected HeLa cells which are not treated with ara-C

The experiments with infected HeLa cells presented above were all performed with cells treated with ara-C to prevent viral DNA replication. These were the conditions in which increased expression of transfected tRNA genes were observed *in vivo* (Berger and Folk, 1985; Gaynor *et al.*, 1985). We also prepared S100 extracts from infected HeLa cells in which DNA synthesis was not blocked with ara-C (Figure 10A). The *in vitro* transcriptional activity of S100 extracts from Ad5-infected HeLa cells dropped dramatically after the period of viral DNA replication (24 and 30 h post-infection). No decrease in activity was observed for extracts of dl312-infected cells in which viral DNA synthesis does not occur. Hoeffler and Roeder (1985) observed a similar decrease in transcriptional activity of nuclear extracts prepared after viral DNA replication. *In vitro* transcription was also performed with nuclei separated from soluble components in an early step of the S100 extract preparation (Figure 10B). As reported earlier (Weinmann *et al.*, 1976), high activity for VAI transcription was observed for nuclei from Ad5-infected cells.

These are the expected results of viral DNA replication based on the properties of stable transcription complexes. When viral DNA replication is not blocked with ara-C, transcription factors would be expected to be sequestered in stable transcription complexes on the replicated viral DNA. Since viral DNA remains associated with the nuclei during preparation of the soluble extract, this would reduce the activity of the extract and yield high VAI transcription in the nuclei, as observed. On the other hand, when viral DNA replication was blocked with ara-C, much less transcriptional activity was sequestered in nuclei, but instead was released into the soluble S100 extract (Figure 10B) as observed earlier. The total level of class III transcriptional activity was increased in Ad5-infected HeLa cells whether or not they were treated with ara-C. When viral DNA synthesis was not blocked with ara-C the increased activity was observed as VAI transcription in isolated nuclei (Figure 10B, lane 6). When viral DNA synthesis was blocked with ara-C, the increased transcriptional activity was observed in the soluble extract (lane 4). Hoeffler

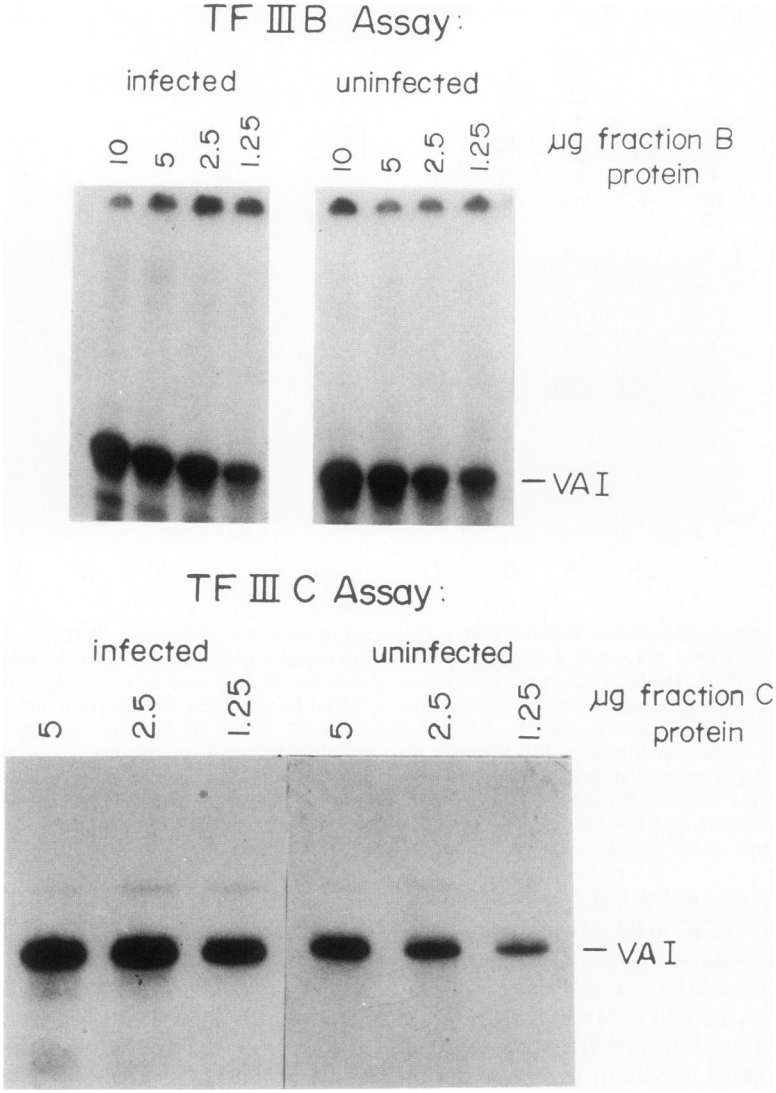

Fig. 7. TFIIIB and TFIIIC titration assays. **Upper panel:** TFIIIB titration. 8 μg of fraction C protein from uninfected HeLa cells and 10 units polIII were added to transcription reactions with 10, 5, 2.5 and 1.25 μg fraction B protein from Ad2-infected or uninfected HeLa cells. An autoradiograph of the reaction products separated by gel electrophoresis is shown. 1 μg Ad5 DNA was used as template. **Lower panel:** TFIIIC titration. 10 μg fraction B protein from uninfected cells and 10 units polIII were added in transcription reactions with 5, 2.5 and 1.25 μg fraction C protein from Ad2-infected or uninfected HeLa cells. An autoradiograph of VAI RNA products is shown. 1 μg of Ad5 DNA was used as template.

and Roeder (1985) also interpreted the decrease in activity of nuclear extracts following viral DNA replication to be due to the stable binding of transcription factors to viral DNA. As further evidence for this, they found high activity of extracts prepared after viral DNA replication when extractions were performed at high salt concentration (1.2 M ammonium sulfate). The high salt extraction may partially or completely disrupt stable transcription complexes formed on viral DNA, permitting recovery of transcription factors in soluble form.

Discussion

These results argue strongly that E1A protein acts indirectly to stimulate the transcription of genes by RNA polymerase III. Extracts from adenovirus-infected HeLa cells were 5 – 10 times more active in the *in vitro* transcription of VAI and tRNA genes than extracts of mock- or E1A mutant-infected HeLa cells (Figures 1 – 3). Although extracts of infected cells contained soluble E1A protein, depletion of E1A protein from these extracts with an anti-E1A protein monoclonal antibody did not decrease activity (Figure 5). Furthermore, phosphocellulose chromatography separated most E1A protein (which flowed through the column in 0.1 M KCl) from factors in the infected cell extract responsible for the higher activity in reconstituted reactions (which eluted between 0.35 and 0.6 M KCl, fraction C; Figures 6 and 7). These results suggest that E1A protein does not stimulate transcription directly, but rather that the expression of E1A protein in infected cells leads to the induction or activation of another factor which stimulates transcription. However, the results might also be explained if minute amounts of E1A protein remaining after immunoprecipitation or present in the phosphocellulose fraction were sufficient to stimulate transcription directly. If this were the case, we would have expected the excess E1A protein present in the unfractionated extract to stimulate transcription when mixed with an extract of uninfected cells. This was not observed. Mixtures of uninfected and infected cell extracts gave the average activity of the two, arguing against the possibility that minute amounts of E1A protein which escaped immunoprecipitation stimulated transcription directly. However, on the basis of these data, we

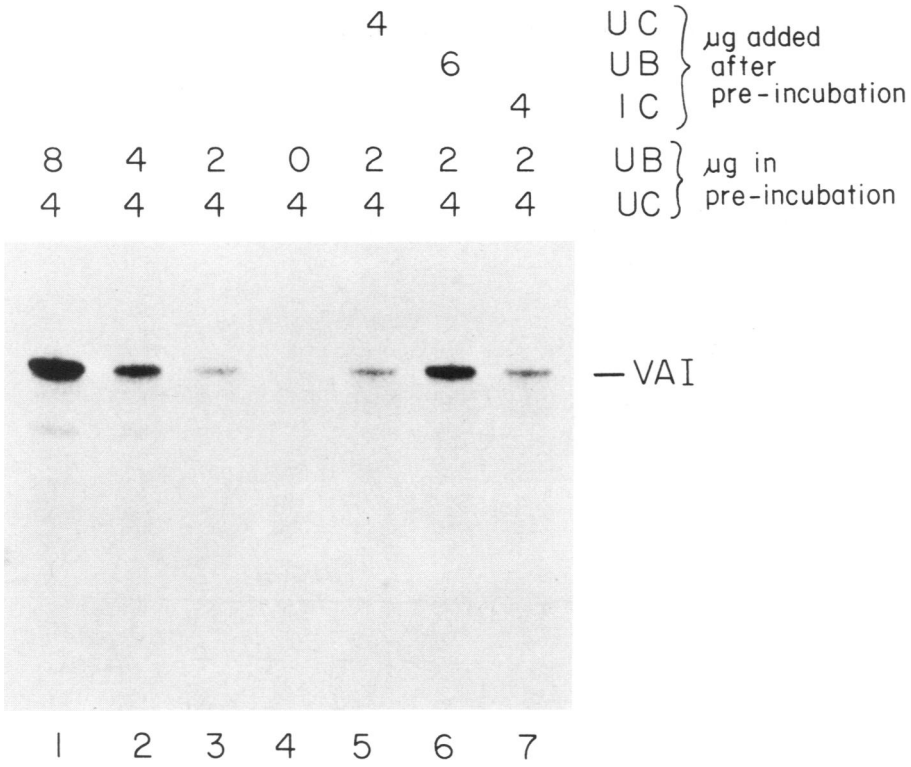

Fig. 8. Fraction C from infected cells does not stimulate transcription from pre-formed stable transcription complexes. 0.5 µg pVA plasmid was incubated in transcription buffer minus nucleoside triphosphates for 10 min with 4 µg phosphocellulose fraction C protein isolated from uninfected HeLa cells (UC) and 8 **(lane 1)**, 4 **(lane 2)**, 2 **(lanes 3,5,6,7)** or 0 µg **(lane 4)** fraction B from uninfected HeLa cells (UB) and 10 units RNA polymerase III. After the 10 min pre-incubation, reactions were initiated by addition of nucleotide triphosphates and terminated after 60 min. No further additions were made for reactions whose products are shown in **lanes 1–4**. After the 10 min pre-incubation, 4 µg UC protein was added to the reaction shown in **lane 5**, 6 µg UB protein to the reaction in **lane 6**, and 4 µg protein from the C fraction isolated from infected cells (IC) was added to the reaction in **lane 7**.

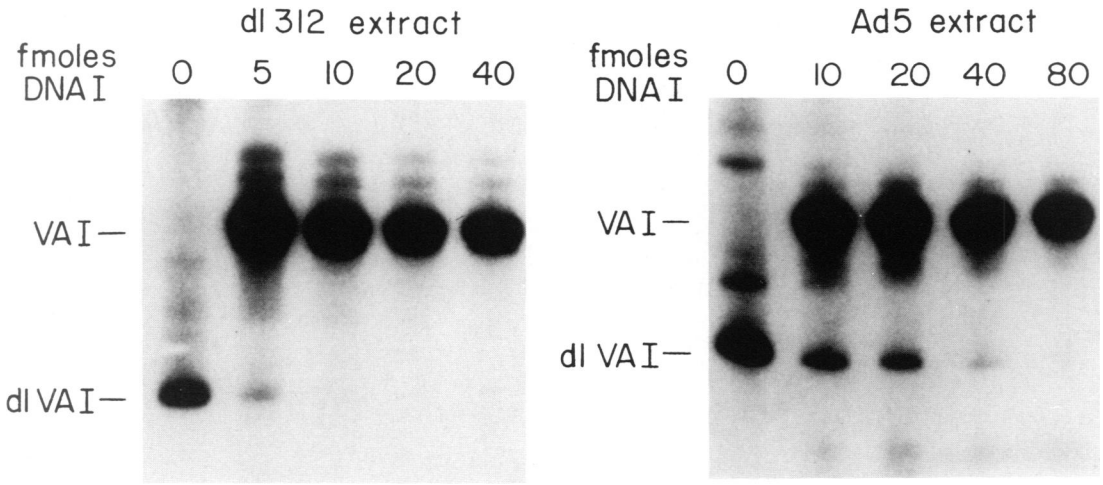

Fig. 9. Template commitment titrations. 10 µg of S100 protein prepared from dl312-(left) or Ad5-(right) infected, ara-C-treated HeLa cells at 30 h post-infection were pre-incubated at 30°C with the indicated amounts of pVAI (DNA I) plus sufficient pBR322 to make a total of 0.5 µg DNA for 40 min. 20 fmol of pdlVAI (DNA II) were then added together with nucleotide triphosphates and incubation continued for 60 min. Autoradiograms of the reaction products resolved by gel electrophoresis are shown. The positions of VAI and dlVAI are indicated. The left autoradiogram was exposed six times longer than the right one.

cannot rule out the possibility that a specifically modified, minor form of E1A protein not detected in the Western blot of Figure 5A might escape immunoprecipitation by the monoclonal antibody, fractionate in the C-fraction on phosphocellulose and stimulate transcription directly.

Hoeffler and Roeder (1985) also reported that phosphocellulose fraction C contains factor(s) chiefly responsible for the higher activity of extracts from adenovirus-infected cells. This fraction also contains TFIIIC (Segall *et al.*, 1980; Lassar *et al.*, 1983), the limiting factor required for class III gene transcription in extracts of uninfected HeLa cells (Fuhrman *et al.*, 1984). This raises the question of whether E1A protein stimulates class III gene transcription indirectly by increasing the activity of TFIIIC. The answer to this question has significant implications for the

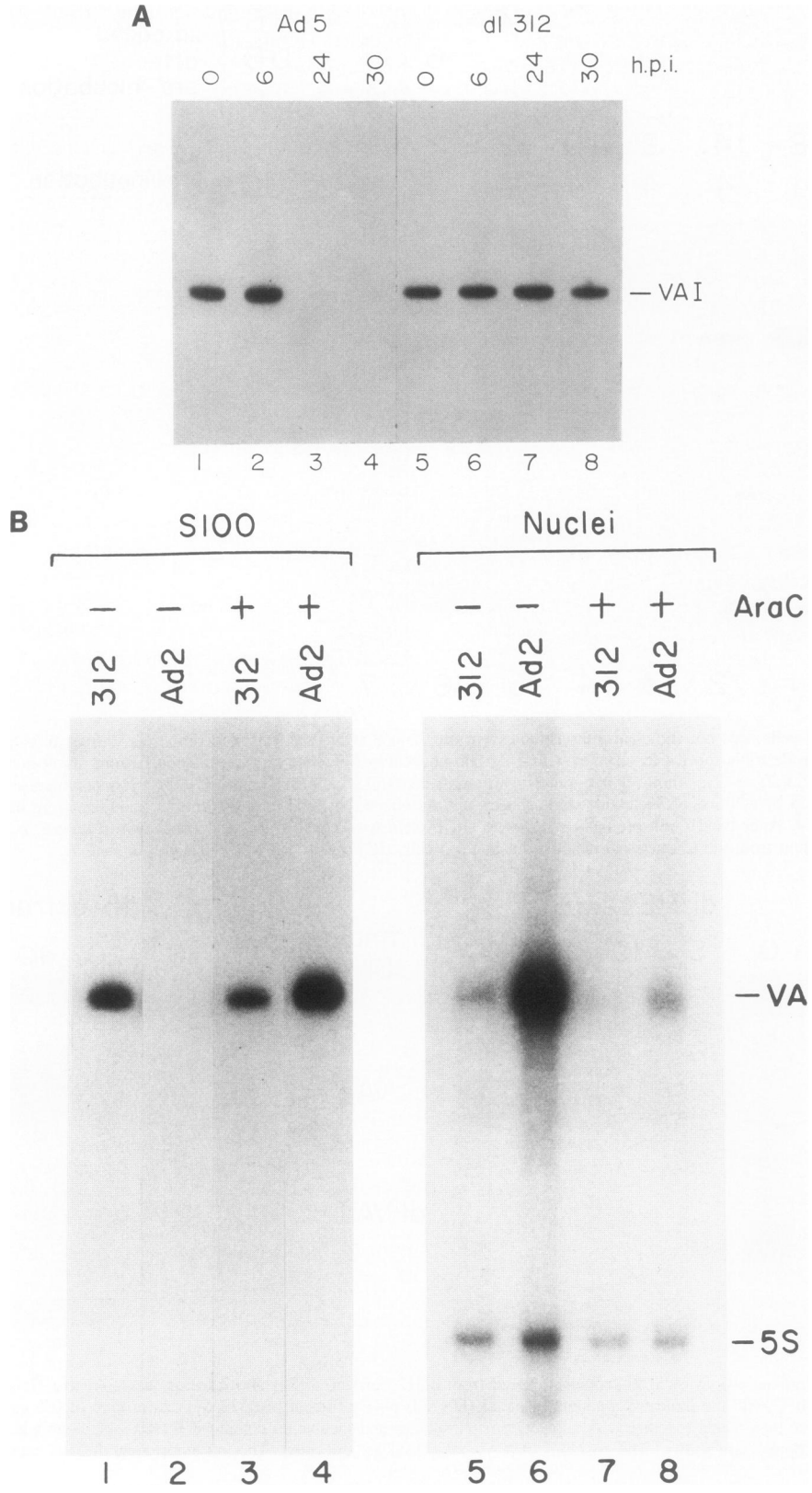

Fig. 10. **(A)** *In vitro* VAI RNA transcription with extracts prepared at various times post-infection of untreated HeLa cells with Ad5 or dl312. S100 extracts were prepared from HeLa cells harvested 0, 6, 24 and 30 h post-infection with Ad5 **(lanes 1–4)** or dl312 **(lanes 5–8)**. 20 μg of extract protein and 1.0 μg Ad5 DNA were used in the transcription reactions. An autoradiograph of reaction products resolved by polyacrylamide gel electrophoresis is shown. **(B)** S100 extracts were prepared from HeLa cells at 30 h post-infection with dl312 **(lanes 1** and **3)** or Ad5 **(lanes 2** and **4)**. HeLa cells were either not treated further **(lanes 1** and **2)** or treated with ara-C from the time of infection **(lanes 3** and **4)**. Products of reactions using 30 μg of S100 protein and 0.75 μg pVA as template are shown. Nuclei were isolated from the same cells during the initial steps in the preparation of the S100 extracts. 5×10^5 nuclei were incubated under the same conditions used for *in vitro* transcription with S100 extracts and the products of these reactions were subjected to electrophoresis and visualized by autoradiography. The positions of VAI and 5S rRNA are indicated.

mechanism of transcriptional *trans*-activation by E1A protein. We performed mixing experiments to search for a stimulatory activity in fraction C other than TFIIIC, but failed to find evidence for it (Figure 8). On the other hand, template commitment titration experiments indicated that extracts of infected cells contained 4–8 times more active TFIIIC molecules than extracts of uninfected cells (Figure 9).

On the basis of these data, we suggest that adenovirus infection leads to higher class III gene transcriptional activity of cell extracts because of an E1A protein-dependent increase in TFIIIC concentration. However, one must be cautious in making quantitative conclusions about complex reactions in crude extracts. While our analyses of S100 extracts indicate that adenovirus E1A functions result in an increase in TFIIIC concentration, other DNA-binding proteins besides TFIIIC present in these preparations could complicate our interpretation of the results. Definitive proof that adenovirus infection results in increased levels of TFIIIC will require direct physical characterization of TFIIIC molecules from infected and uninfected cells. Such an analysis awaits the purification of TFIIIC.

With these qualifications in mind, it is interesting to consider the model that E1A expression results in an increase in TFIIIC concentration. An increased concentration of TFIIIC would be expected to stimulate transcription in HeLa cell extracts, since TFIIIC is the limiting factor required for tRNA and VAI gene transcription in S100 extracts of uninfected HeLa cells (Fuhrman *et al.*, 1984). If TFIIIC is the limiting factor required for transcription of transfected genes *in vivo*, as it is in S100 extracts (which are prepared near physiological salt concentrations), then an increase in TFIIIC concentration *in vivo* would increase the transcription of transfected genes, as observed (Berger and Folk, 1985; Gaynor *et al.*, 1985; Hoeffler and Roeder, 1985).

The increased transcription of class III genes in Ad5-infected, ara-C-treated HeLa cells was only observed for transfected tRNA genes. The transcription of endogeneous tRNA and 5S rRNA genes in these cells was not greatly increased by Ad5 infection (Gaynor *et al.*, 1985). These observations can also be explained by an increase in TFIIIC concentration. The explanation depends on recent findings for the control of *Xenopus* 5S rRNA genes. Brown and co-workers (Bogenhagen *et al.*, 1982; Schlissel and Brown, 1984; Brown, 1984) have shown that 5S rRNA genes which are inactive *in vivo*, are in chromatin structures which sterically block interactions with transcription factors. In contrast, 5S genes which are active *in vivo* were shown to be stably associated with transcription factors. If all class III genes were in either repressed chromatin structures or stable transcription complexes like *Xenopus* 5S rRNA genes, an increase in TFIIIC concentration would not affect their transcription. The increased numbers of TFIIIC molecules would still be sterically blocked from interacting with genes in repressed chromatin structures. Stable transcription complexes would already include bound TFIIIC, and their transcription would not be stimulated by an increase in the concentration of free TFIIIC. On the other hand, transfected tRNA genes would be able to interact with an increased number of TFIIIC molecules, resulting in an increased number of genes assembled into active transcription complexes and therefore the observed increase in transcription. In this way, an increase in the *in vivo* concentration of free TFIIIC would account for the increased transcription of transfected tRNA genes without causing an increase in the transcription of endogenous class III genes.

Transfection (Weeks and Jones, 1983; Imperiale *et al.*, 1983) and microinjection (Ferguson *et al.*, 1984) studies have shown that E1A and IE proteins can act independently of other viral proteins to stimulate transcription of early adenovirus genes by polII *in vivo*. The findings presented in this paper prompt us to suggest the model that E1A and IE proteins stimulate transcription of these genes by increasing the activities of key cellular class II transcription factors which are the limiting components required for their transcription in uninfected cells. This model would explain the ability of E1A and IE proteins to stimulate transcription of non-homologous viral and cellular genes. Those genes, viral and cellular, whose transcription is stimulated by E1A and IE proteins, would be genes whose transcription in uninfected cells is limited by the same cellular transcription factors which are induced or activated by E1A and IE proteins. The model would also explain why mutations in the early region E2A and E3 promoters depress transcription to the same extent from the high transcription level in cells containing E1A protein and the low basal transcription level observed in cells which do not contain E1A protein (Elkaim *et al.*, 1983; Kingston *et al.*, 1984; Murthy *et al.*, 1985; Imperiale *et al.*, 1985). The implication of the promoter mutagenesis studies is that the same DNA-protein contacts are required for the high level of transcription in cells expressing E1A protein and for the lower level of transcription in cells without E1A proteins. The model would account for this since the same transcription factors would interact with the early promoters whether or not E1A protein is expressed. However, the activity of the limiting transcription factors would be increased by E1A protein, stimulating transcription by increasing the number of viral DNA molecules assembled into transcription complexes. Further analyses of both class II and III transcription factors in uninfected and adenovirus-infected cells will be needed to test these hypotheses.

Materials and methods

Cell culture and viruses

HeLa cells were maintained in suspension culture in MEM with 5% newborn calf serum. 293 cells were maintained in suspension culture in MEM with 5% fetal calf serum. Ad2 and Ad5 were propagated in HeLa suspension cultures and titers determined by plaque formation on HeLa cell monolayers. Mutant dl312 (Jones and Shenk, 1979a) was propagated on 293 cells in suspension and titer determined by plaque formation on 293 cell monolayers. Cells were infected with virus at a MOI of 50. Where indicated, ara-C was added to HeLa cell cultures to 20 μg/ml at the time of infection and every 8–12 h thereafter.

Extract preparation

S100 protein extracts were prepared as described by Wu (1978) and Weil *et al.* (1979) with the following modifications. Cells were harvested and washed three times with wash buffer [30 mM Hepes, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT) and 20% (v/v) glycerol]. Two times the packed cell volume of hypotonic buffer (10 mM Hepes, pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$ and 0.5 mM DTT) was added and the cells were allowed to swell for 15 min on ice. Cells were disrupted in a Kontes all-glass Dounce homogenizer by 12–25 strokes with a B pestle. Cell lysis was >95% and nuclear lysis was minimal. One tenth volume of 10 × isotonic buffer (300 mM Hepes, pH 8.0, 1.27 M KCl, 40 mM $MgCl_2$, 50 mM DTT) was added and the lysate was centrifuged at 100 000 *g* for 60 min. For analysis of *in vitro* transcription in nuclei, nuclei were first removed by centrifugation at 1200 r.p.m. in an IEC DPR 6000 for 5 min before the high speed centrifugation. Glycerol was added to the supernatant to 20%. As found previously (Wu, 1978; Weil *et al.*, 1979), S100 extracts could be stored for many months at −70°C without significant loss in activity.

Transcription reactions

Transcription reactions were in 40 μl of 30 mM Hepes, pH 8.0, 3 mM DTT, 8.25 μg/ml creatine phosphokinase, 5 mM creatine phosphate, 1 μg/ml α-amanitin, 500 μM each ATP, CTP and UTP and 25 mM GTP with 2 μCi [α-^{32}P]GTP (Amersham). S100 extract, phosphocellulose fractions A, B, C and D, polIII and template DNA were added as indicated. Template DNA was either Ad5 DNA, the plasmid pVA containing Ad2 sequences from 10 589 to 11 555 cloned between the *Sal*I and *Hind*III sites of pBR322 (Guilfoyle and Weinmann, 1981), or pdlVAI, a pBR322 clone of the Ad2 VAI gene with a deletion of nucleotides

73–124 in the VAI RNA sequence (Fowlkes and Shenk, 1981). Ten units of partially purified polIII were added to reactions with phosphocellulose fractions. RNA polIII was purified from HeLa cells by the method of Jaenhing *et al.* (1977) through DEAE-Sephadex (replacing DEAE-cellulose), heparin-agarose (replacing heparin-Sepharose), DEAE-cellulose, DEAE-Sephadex and the first phosphocellulose column in the published procedure. The preparation was free of detectable TFIIIB or TFIIIC activity. 10 units of polIII was in excess when used in transcription reactions with 10 μg fraction B and 4 μg fraction C. Unless otherwise indicated, incubations were for 90 min at 30°C and reactions were terminated by addition of SDS to 0.5% and EDTA to 12 mM. *In vitro* transcription in isolated nuclei was performed under the same reaction conditions as for S100 extracts, except that template DNA was replaced with 5×10^5 nuclei per reaction. Following addition of 20 μg carrier tRNA, RNA extraction buffer (3.5 M urea, 0.175 M NaCl, 5 mM Tris, pH 7.4, 5 mM EDTA, 0.5% SDS) was added to 400 μl total volume. The mixture was phenol-chloroform extracted twice and ethanol precipitated. RNA was resolved by electrophoresis on 8% polyacrylamide gels containing 8 M urea. Gels were soaked for 30 min in cold 5% trichloroacetic acid (TCA) to remove urea, rinsed with water, dried and exposed to autoradiographic film. The synthesis of specific transcription products was quantitated by cutting bands from dried gels and counting Cerenkov c.p.m. directly in toluene-based scintillation fluid. Incorporation of [α-^{32}P]GTP was calculated from the known specific activity of the label obtained by counting an aliquot of the total transcription mix. The molar yield of specific transcripts was calculated by dividing the molar incorporation of GTP by the number of guanine residues in the transcript (52 for VAI RNA).

Depletion of E1A proteins from S100 extracts by immunoprecipitation

25 μl of S100 extracts were incubated with 25 μl (packed volume) of monoclonal antibodies covalently coupled to Sepharose for 3 h on ice with stirring every 15 min. Anti-E1A protein monoclonal antibody R7 is a rat IgG purified from supernatants of serum-free medium by ammonium-sulfate precipitation (A.Tsukamoto and A.Berk, in preparation). Anti-ICP4 was mouse IgG directed against the ICP4 protein of herpes simplex virus type I, kindly provided by Larry Feldman. Monoclonal antibodies were coupled to CNBr-activated Sepharose by the method recommended by Pharmacia. Control extracts were incubated with 25 μl of CNBr-activated Sepharose taken through the coupling procedure in the absence of protein. Immunoprecipitates were removed from the extract by centrifugation in an Eppendorf microcentrifuge for 10 s. The supernatant was removed and subjected to two more 10-s centrifugations. The entire procedure was then repeated so that each extract was taken through two cycles of immunoprecipitation. The protein concentration of the final supernatant was determined, and 20 μg of immunoprecipitated S100 extract protein was used in the *in vitro* transcription reactions shown in Figure 5B. Western immunoblot analysis was performed on equivalent aliquots of the final supernatants and on a 4 M NaSCN extract of the combined immunoprecipitate pellets.

Phosphocellulose fractionation

S100 extracts were fractionated by chromatography on phosphocellulose according to the procedure of Segall *et al.* (1980). The S100 extract was dialyzed against Buffer A (20 mM Hepes, pH 7.9, 0.2 mM EDTA, 0.5 mM DTT and 20% glycerol) with 100 mM KCl. The phosphocellulose column was loaded with 10 mg S100 protein per ml bed volume. The flow-through fraction and successive step elutions of 0.35 M, 0.6 M and 1.0 M KCl were designated fractions A, B, C and D, respectively. The fractions were dialyzed against Buffer B (Buffer A, plus 5 mM $MgCl_2$, 100 mM KCl). Transcription reactions with individual fractions plus 10 units of polIII indicated minimal cross-contamination of TFIIIC activity in B fractions, or of TFIIIB activity in C fractions. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard.

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