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# Specific transcription from the adenovirus E2E promoter by RNA polymerase III requires a subpopulation of TFIID

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## ABSTRACT

**The early E2 (E2E) promoter of adenovirus type 2 possesses a TATA-like element and binding sites for the factors E2F and ATF. This promoter is transcribed by RNA polymerase II in high salt nuclear extracts, but by RNA polymerase III in standard nuclear extracts, as judged by sensitivity to low and high, respectively, concentrations of α-amanitin. Transcription by the two RNA polymerases initiated at the same site and depended, in both cases, on the TATA-like sequence and upstream elements. However, RNA polymerase III transcripts, unlike those synthesized by RNA polymerase II, terminated at two runs of Ts downstream of the initiation site. Although they are not essential, sequences downstream of the initiation site increased the efficiency of E2E transcription by RNA polymerase III. Such RNA polymerase III dependent transcription required a subpopulation of the general transcription factor, TFIID: TFIID that binds weakly to phosphocellulose (0.3 M eluate) complemented a TFIID-depleted extract to restore RNAp III transcription, whereas TFIID tightly associated with phosphocellulose (1 M eluate) was unable to do so.**

## INTRODUCTION

In eukaryotic cells, three distinct RNA polymerases synthesize RNA. RNA polymerases I, II, and III, respectively, transcribe genes encoding ribosomal RNA, pre-messenger RNA, and various short RNAs including transfer RNAs and 5S ribosomal RNA. RNA polymerase III (RNAp III) also synthesizes the small nuclear RNA, U6, and several other small RNA species that are components of ribonucleoprotein complexes, such as 7SK, H1, and MRP RNAs (1). The selectivity with which RNA polymerases are recruited for transcription seemed to be a function of promoter configuration. Early data, for example, indicated that promoters of class II genes comprise elements upstream of the transcription initiation site, whereas class III genes utilize intragenic promoters. The TATA element, located 25–30 bp upstream of sites of initiation, and present in the majority of class II genes, appeared to be an exclusive characteristic of this class. This element is the binding site for the general transcription factor, TFIID, which has been demonstrated to be essential for the transcription of class II genes (2). Furthermore, the protein factors found to interact with upstream sequences were believed to be class II specific factors, as their cognate sequence elements had not been found in class III genes. This latter class was distinguished by intragenic DNA sequence motifs (A and B), which are responsible for binding TFIIIC, the general class III factor, thought to be essential for RNAp III initiation (1).

The discovery of a group of class III genes that possess an architecture very similar to class II genes has shown that the lone criterion of promoter primary structure, as represented by a linear array of modular sequence elements is insufficient to explain RNA polymerase selectivity. The genes for U6, 7SK, 7SL, H1, MRP and EBER all possess upstream promoter elements that bind factors that stimulate RNAp II transcription (3–5). Among such upstream elements is a TATA sequence, located, as in class II genes, about 25 bp from the sites of initiation. This element is absolutely essential for RNAp III transcription of the U6 gene (6,7), the best characterized member of this group. Paradoxically, deletion of the TATA element results in recognition of the U6 promoter by RNAp II (7). Furthermore, TFIID has been shown to be required not only for transcription of the U6 gene (8–10), but also for all class III genes (11–13). Taken together, these observations indicate that there must be some subtle mechanistic differences between initiation of an RNA chain by RNAp II or RNAp III. *In vitro* studies of the c-*myc* promoter accentuate this point (14): transcription of this gene can be initiated by either RNAp II or RNAp III. The two polymerases appear to transcribe RNAs with identical 5′ ends, but with different 3′ ends. This phenomenon was observed at both the P1 and P2 promoters of the gene, and when the gene was micro-injected into oocytes (15). We now report another example of a promoter that can be recognized by RNAp II and RNAp III: both polymerases can initiate transcription from the promoter of the E2E gene of adenovirus type 2.

The E2 transcription unit of adenovirus subgroup C (types 2 or 5) is activated by E1A proteins during the early phase of infection (see 16). Transcripts of the E2 transcription unit are processed to generate an abundant mRNA encoding the 72 kD single stranded binding protein and much lower levels of the DNA polymerase and pre-terminal protein mRNAs (see 16). A number

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of studies of the early E2 (E2E) promoter have identified several sequence elements that are important for its efficient transcription (17–19). A TATA-like sequence, (Figure 1) located some 25 bp upstream of the major initiation site is critical for efficient transcription from the +1 site *in vitro* and in infected cells (17–20). Three additional upstream DNA sequence elements (Figure 1), a pair of E2F binding sites and an ATF binding site, greatly stimulate transcription (18–21). Mutation of any one of these sequence elements reduces the level of transcription *in vivo*. *In vitro* studies using reconstituted components have demonstrated that purified ATF protein is able to stimulate transcription from the E2E promoter (22).

We now report that E2E transcription by RNAp III depends on upstream elements defined as components of the RNAp II promoter. The similarity of the requirements for the transcription of this promoter by the two polymerases underscores the issue of how a particular type of polymerase is recruited. To begin to address the mechanism of polymerase selectivity, we have examined the requirement for specific TFIID complexes in transcription from the E2E promoter by RNAp III.

## MATERIALS AND METHODS

### Nuclear extracts and phosphocellulose fractions

Nuclear extracts for RNA polymerase III transcription were prepared from HeLa cells essentially as described by Dignam *et al.* (25). Nuclei were resuspended in 1.5 times the original packed cell volume in a buffer containing 20 mM HEPES (pH 7.6), 1.5 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 20% glycerol, and 1 mM PMSF. The total volume was measured, and concentrated NaCl was added to yield a final concentration of 0.3 M. After stirring for 30 min at 4°C, the nuclei were pelleted at 25,000×g and the supernatant was dialyzed against 20 mM HEPES (pH 7.6), containing 2 mM DTT, 0.1 mM EDTA and 20% glycerol (buffer A) supplemented with 0.1 M KCl. Following dialysis, insoluble material was removed by centrifugation at 25,000×g. Protein concentrations were generally about 5 mg protein per ml of extract. Nuclear extracts used for RNA polymerase II transcription were prepared according to the protocol of Shapiro *et al.* (24), except that insoluble material was removed following the final dialysis as described above. These nuclear extracts yielded protein concentrations of 10–12 mg/ml as determined by the method of Bradford (44), using bovine serum albumin as the standard.

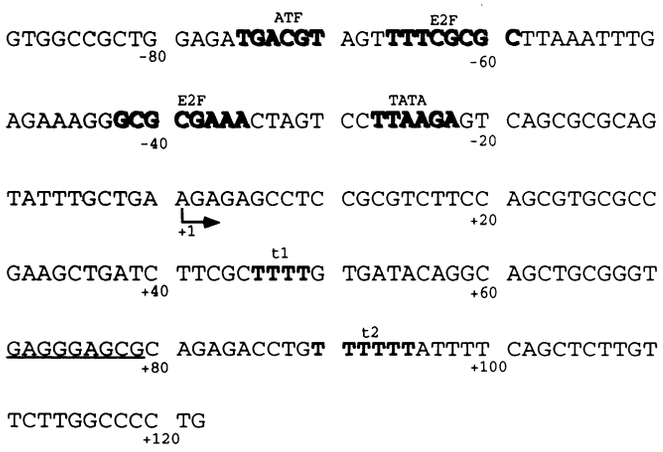


**Figure 1.** The structure of the E2E promoter. The noncoding strand of the Ad2 E2E promoter is presented. The arrow depicts the major initiation site for RNA II transcription, as defined by cap analysis of *in vivo* RNA (47). The sequences of the previously characterized upstream promoter elements have been highlighted. The putative RNA polymerase III termination sites (bp +46 to +49 and bp +90 to +95) are shown in bold type. A downstream sequence (bp +71 to +79) that has an exact match to a sequence downstream of the c-*myc* P2 promoter is indicated by underlining. The S1 oligonucleotide probe used to assay the transcription reactions is a 60 nt fragment complementary to the strand shown, extending from nt −15 to nt +45, end-labelled at its 5′-end.

Phosphocellulose fractions were obtained from nuclear extracts as described (34). Extracts were applied to phosphocellulose resin that had been equilibrated in buffer A containing 0.1 M KCl, at a concentration of 10 mg of protein per ml of packed resin. The flow-through fraction was collected (fraction A), and steps of 0.3 M KCl (fraction B), 0.5 M KCl (fraction C), and 1 M KCl (fraction D), all in buffer A, were then used to elute the bound material. The three bound fractions were dialyzed against buffer A containing 0.1 M KCl.

### Immunodepletion of nuclear extract and fractions

Bacterially expressed, human TATA-binding protein (TBP) was used as the antigen to prepare monoclonal antibodies. Hybridomas were screened by probing blots to which the phosphocellulose 1 M fraction from HeLa cell nuclear extracts had been transferred following electrophoresis in SDS-polyacrylamide gels (48). The clone used (MBP-6), recognizes an epitope in the N-terminal 100 amino acids of TBP (49). Monoclonal antibody supernatants were purified on protein-A-Sepharose and used for batch depletions. A saturating amount of purified antibody was added to nuclear extracts and the mixture incubated at 0°C for 1 hr. Protein A-Sepharose (50% suspension in buffer A containing 0.1 M KCl) was added, and the mixture incubated for an additional hour with occasional mixing. The immobilized material was removed by a 2 min spin in an Eppendorf microfuge, and the supernatants were used for transcription. A purified monoclonal antibody specific for E1A (M73) (45) was used in parallel as a control. Alternatively, the antibodies were cross-linked to the protein-A-Sepharose (43), and the depletions were performed by passing the extract or fraction several times over an antibody column. Dilutions using either method were estimated to be between 10 and 20%, and were compensated for in transcription reactions. Antibody columns were washed with 10 column volumes of a buffer containing 20 mM HEPES, pH 7.8, 0.2 M KCl, and 0.1 mM EDTA. The column was eluted with the same buffer containing 2 M urea and dialyzed against buffer A containing 0.1 M KCl. Immunoblots probed with excess anti-TBP antibody were used to quantitate the amount of TFIID in the various fractions.

### Templates used for transcription

The wild type templates used for transcription were either pE2, which comprises a *Nae* I–*EcoR* I fragment of adenovirus-2 (bp 26,493–27,372) inserted into pUC19 between the *Sma* I and *EcoR* I sites, or pEII, which is an *Nar* I fragment (bp 26,893–27,187) inserted into pSP73 at the *Cla* I site. Deletions or linker substitutions were derived from one of these two plasmids. The upstream mutants were based on those described by Murthy *et al.* (18) and were introduced into the pEII background. One minor modification was the elimination of a base pair insertion in their linker mutant, −19/−29. A double E2F linker mutant was constructed using the −55/−66 mutant of Murthy *et al.* (18), and included the −36/−45 mutation

described by Loecken and Brady (21). Downstream deletion mutants (+151 and +117) were constructed using appropriate primers for the polymerase chain reaction, and subsequently subcloned into pUC19 at the *Sma* I and *EcoR* I sites. The +62 mutant comprised a *Pvu* II to *EcoR* I fragment (Ad2 bp 27,030−27,372) subcloned into pUC19 at the *Sma* I and *EcoR* I sites. All plasmids used as templates were purified as described (46) and added to transcription reactions in circular form.

### Transcription reactions

Reaction mixtures (30 μl) contained 20 mM HEPES-KOH buffer, pH 7.9, 6 mM $MgCl_2$, 2 mM DTT, 600 μM each nucleotide triphosphate, and the concentrations of template and nuclear extract indicated in figure legends. Mixtures were incubated for 1 hr at 30°C, adjusted to 0.8 M ammonium acetate, 0.1% SDS, 2 mM EDTA, and *Escherichia coli* tRNA (10 μg) was added. The mixture was diluted to a final volume of 300 μl, extracted with phenol-chloroform (300 μl, 1:1), chloroform (300 μl), and precipitated with 800 μl of ethanol. Samples were resuspended in 10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 40 mM NaCl (25 μl), DNase I was added (1.5 μg), and the the reactions were incubated for 20 min at 37°C. The samples were deproteinized as described above, 20 fmol (12,000 cpm/fmol of probe) of an oligonucleotide probe (Figure 1) were added and the samples were ethanol precipitated. The precipitated samples were washed with 80% ethanol, air dried for 30 min, and resuspended in (20 μl) 80% formamide, 40 mM Pipes-NaOH, pH 6.4, 400 mM NaCl, 1 mM EDTA. Following incubation for 10 min at 70°C, samples were incubated overnight at 30°C. Nuclease S1 was added (125 units) in a buffer (300 μl) containing 50 mM sodium acetate, pH 4.5, 0.28 M NaCl, 4.5 mM $ZnSO_4$ and the mixtures were incubated for 1 hr at 30°C. The reaction was adjusted to 0.8 M ammonium acetate, 4 mM EDTA, *Escherichia coli* tRNA (3 μg) was added, and the mixtures were precipitated with 1 ml of ethanol. The products were resuspended in 80% formamide (6 μl) and analyzed by electrophoresis in 10% polyacrylamide (1:19 bisacrylamide) gels containing 8.3 M urea, 90 mM Tris, 90 mM Borate, and 2 mM EDTA. Gels (45×15×0.04 cm) were run at 1800 volts until the bromophenol blue dye reached the bottom. After drying and autoradiography, products were excised and quantitated by counting in the presence of scintillation fluid. Infected cell, cytoplasmic RNA made by deproteinization of NP-40 solublised extracts was used as a marker for E2E initiation sites.

When transcripts were analyzed by ribonuclease protection, the procedure was the same as for S1 analysis with the following modifications; hybridization using an RNA probe (330,000 cpm/pmol guanosine) was carried out at 37°C, T1 ribonuclease was added (1000 units, Worthington Biochemical) in a buffer (350 μl) containing 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 5 mM EDTA, and digestion was carried out for 30 min at 30°C. Reactions were terminated by the addition of 10% SDS (20 μl), and proteinase K (50 μg), incubated for 15 min at 37°C, and extracted with phenol-chloroform (400 μl, 1:1) and precipitated with 1 ml of ethanol. The products were analyzed by electrophoresis in 6% polyacrylamide gels.

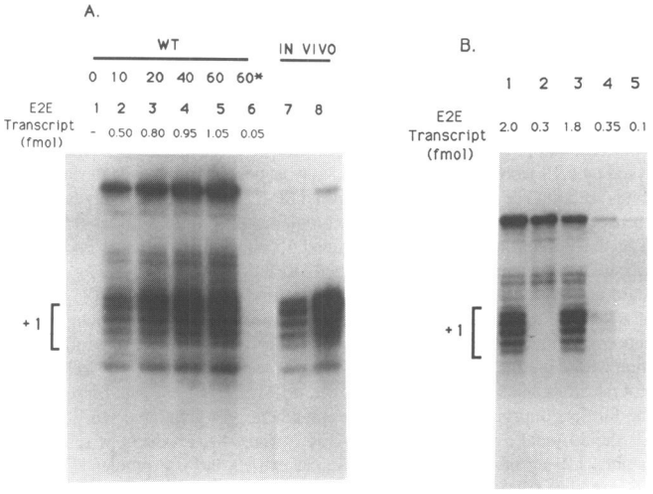


**Figure 2.** RNA polymerase II dependent transcription of the adenovirus E2E promoter in 'high salt' nuclear extracts from HeLa cells. Transcription reactions were carried out as described in the Materials and Methods section. 0.8 pmol of pE2 wild type template was incubated with high salt nuclear extracts (140 μg) for 1 hr at 30°C. The products were analyzed by an S1 nuclease protection assay, using a 5′ end-labelled oligonucleotide probe complementary to the E2E region from bp +45 to −15. Correctly initiated RNA is represented by the bracketed bands labelled +1. **A**, Portions (30 μl) were removed at the times indicated (min) from a reaction mixture that had been scaled-up 8-fold, and mixed with an equal volume of SDS (1%) and EDTA (20 mM) to stop the reaction. The reaction whose products are shown in lane 6 contained 2 μg/ml of α-amanitin. Lanes 7 and 8 show 10 μg and 20 μg, respectively, of cytoplasmic RNA extracted from HeLa cells 11 hr after infection with adenovirus 5 analyzed by the same S1 protection assay. **B**, Transcription, as described for panel A, was carried out with the following templates: wild type (lane 1), TATA mutant (−19/−29) (lane 2), double E2F mutant (−36/−45, −55/−66) (lane 3), and ATF mutant (−74/−85) (lane 4). Lane 5 shows products of a reaction containing the wild type template carried out in the presence of 2 μg/ml of α-amanitin.

## RESULTS

### RNA polymerase II specific transcription in high salt nuclear extracts

It has previously been demonstrated that accurate initiation of E2E transcription in HeLa cell extract requires RNAp II, and is stimulated by the TATA element, as well as the ATF binding site (22,23,17). It was important to establish that our *in vitro* system correctly initiated transcription and was dependent upon these upstream sequences. We initially used a nuclear extract prepared in the manner described by Shapiro *et al.* (24). This procedure involves extraction of the nuclei with approximately 0.4 M ammonium sulfate, followed by concentration of the soluble proteins by precipitation with ammonium sulfate. When these nuclear extracts were incubated with a plasmid containing the E2E promoter, and the resulting transcription products analyzed by S1 nuclease protection using an oligonucleotide probe, increasing amounts of correctly initiated transcripts were observed as a function of time (Figure 2A). Synthesis of E2E transcripts was sensitive to 2 μg/ml of α-amanitin, indicative of RNAp II transcription, and their 5′ ends were coincident with those of authentic, *in vivo* transcripts analyzed by the same assay (Figure 2A). In order to confirm that initiation of E2E transcription in this system required the upstream sequence elements previously identified for this promoter, we used several linker substitution mutations based on those previously shown to impair production of E2E mRNA *in vivo* (18−20). The TATA element mutation reduced E2E transcription to near background levels (Figure 2B, lane 2), whereas mutation of the ATF binding site decreased the efficiency of transcription about 5-fold (Figure 2B, lane 4). A double mutation altering both E2F binding sites had little effect on transcription under these conditions

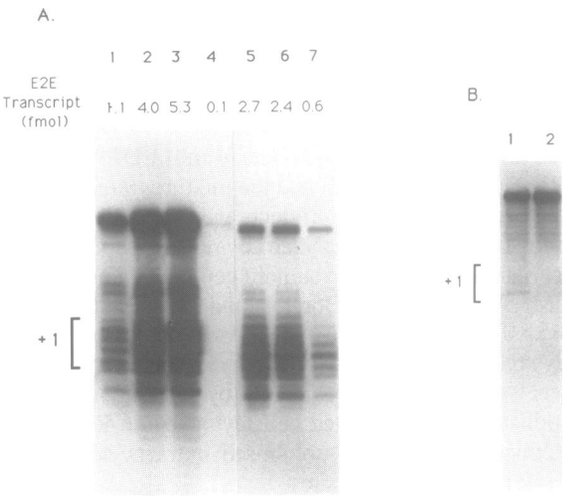


**Figure 3.** Both α-amanitin sensitive and α-amanitin resistant transcripts can be initiated from the E2E promoter. Transcription reactions were carried out as described in the Materials and Methods section. **A**, 0.4 pmol of pE2 wild-type template was used, and reactions were incubated for 1 hr at 30°C. Lanes 1−4 show results of a titration, 4 μl, 8 μl, 12 μl and 12 μl respectively, of high salt nuclear extract (11.8 mg protein/ml). The reaction whose products are shown in lane 4 contained 2 μg/ml of α-amanitin. The reactions whose products are shown in lanes 5−7 transcription were carried out under identical conditions using 60 μg of the standard nuclear extract and contained 0, 2 or 150 μg/ml α-amanitin (lanes 5−7, respectively). **B**, transcription conditions were identical to those described for panel A, but contained 0.4 pmol of a plasmid containing the adenovirus major late promoter (Ad MLP). The reaction whose products are shown in lane 2 contained in addition, 2 μg/ml of α-amanitin. The products were analyzed by S1 nuclease analysis, using a 5′ end-labelled oligonucleotide probe complementary to bp +46 to −15 of the Ad MLP.

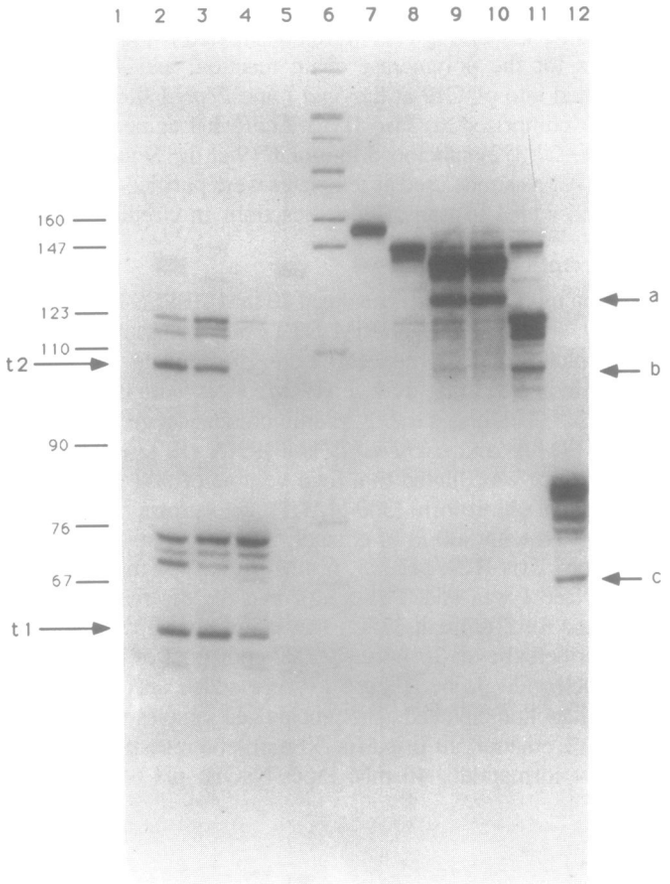


**Figure 4.** The α-amanitin resistant transcripts terminate at one of two downstream runs of T-residues. Transcription reactions were performed and the products were analyzed by a T1 ribonuclease protection assay as described in the Materials and Methods section. The RNA probe used was an SP-6 generated, internally labelled anti-sense probe (330,000 cpm/pmol G) complementary to nucleotides +120 to −16 of the E2E promoter. It also contained 15 nucleotides at its 5′ end from the plasmid from which it was synthesized. Reactions contained 44 μg of the standard nuclear extract, 2 μg/ml of α-amanitin and no template (lane 1) or 0.4 pmol wild-type template (lanes 2 and 5), a template containing downstream sequences to either position +117 (lane 3), or to position +62 (lane 4). The reaction whose products are shown in lane 5 was incubated at 0°C. The following control reactions and size markers are shown. pBR322 DNA cut with Msp I (lane 6); the full length probe, 152 nt (lane 7); a synthetic, T7 generated sense RNA, which should yield a protected RNA of 136 nt (lane 8). Products of reactions containing 140 μg of the RNAp II-dependent extract and 0.8 pmoles of wild-type E2 template (lane 9 and 10), of a template containing a 10 bp deletion between positions +91 and +100, Δ(91−100). (lane 11) or of a template extending only to position +62 (lane 12) are also shown. The sizes expected for correctly initiated, read-through transcripts synthesized by RNA polymerase II are: wild-type, 124 nt, Δ(91−100), 104 nt, and +62, 66 nt. Transcripts initiating upstream of the +1 site are seen as a cluster of more slowly migrating of bands, because the 3′ end of the probe comprises the sequence in GCGCG.

(Figure 2B, lane 3), suggesting that in these HeLa extracts E2F cannot accelerate the formation of active initiation complexes.

## Polymerase III can initiate transcription from the polymerase II initiation site

Extracts that supported mainly RNAp III transcription were prepared by extracting nuclei with 0.3 M NaCl. When such extracts were used to transcribe the adenovirus type 2 major late promoter (MLP), only low levels of specific ML transcription, but high non-specific transcription of the plasmid were observed (Figure 3B, lane 1). In the presence of 2 μg/ml α-amanitin to inhibit RNAp II, only non-specific transcription was detected (Figure 3B, lane 2), indicating that RNAp III cannot specifically initiate ML transcription. By contrast, when these same extracts were used to transcribe the E2E promoter, α-amanitin resistant transcription from the same initiation site as that used by RNAp II was observed (Figure 3A, lanes 5 and 6). Addition of a higher concentration of α-amanitin (150 μg/ml), eliminated most of the transcription (Figure 3A, lane 7). This observation suggested that RNAp III could recognize the E2E promoter and accurately initiate synthesis of a transcript, and indicated that RNAp III transcription is promoter specific.

RNA polymerase III is known to terminate transcription at runs of 4 or more T residues in the non-coding strand of the template (26). Two such sequences, 4 Ts beginning at +46 and a stretch of Ts at +90, are present downstream of the E2E initiation site (Figure 1). If RNAp III were initiating transcription from the E2E promoter, then one would expect to observe products generated by termination at such a stretch of Ts downstream of the initiation site. The S1 probe used to assay initiation was an end-labelled oligonucleotide that extended to position +45 relative to the major E2E initiation site (Figure 1), and would not have differentiated such terminated transcripts from longer ones. In order to determine if such transcripts were indeed produced in these *in vitro* reactions, a probe that included sequences to +120 was employed. A more convenient T1 ribonuclease protection assay was adopted, using an SP-6 generated, single stranded RNA probe that comprised antisense E2E sequences from −16 to

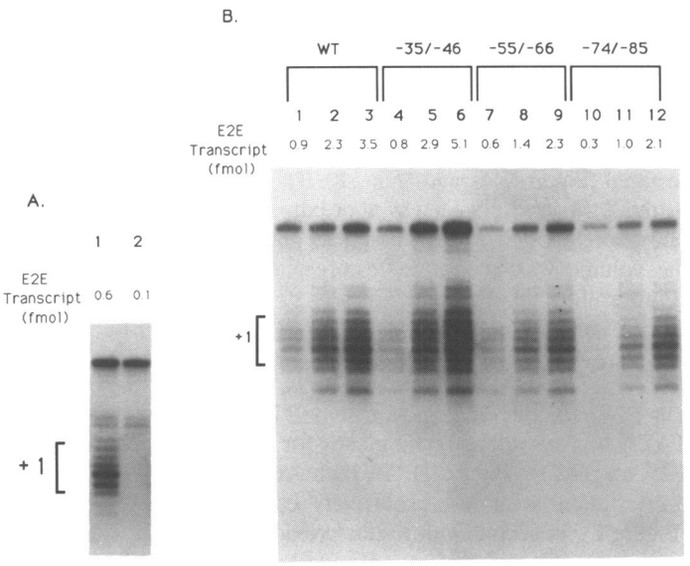


**Figure 5.** RNA polymerase III is stimulated by the same upstream elements as used by RNA polymerase II. Transcription reactions were carried out as described in the Materials and Methods section. Reactions contained 44 μg of standard nuclear extract, the indicated concentrations of template, and were carried out in the presence of 2 μg/ml of α-amanitin. **A**, 0.4 pmol of the wild-type template (lane 1) is compared with the same amount of the TATA (−19/−29) mutant (lane 2). **B**, A titration (0.1, 0.2, 0.4 pmol) comparing templates carrying the following upstream mutations: wild-type (lanes 1−3), −35/−46 (lanes 4−6), −55/−66 (lanes 7−9), and −74/−85 (lanes 10−12).

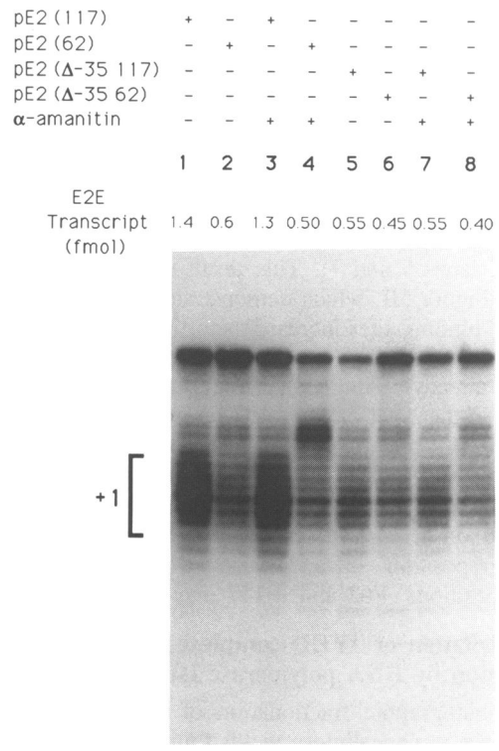


**Figure 6.** Sequences downstream of of the E2E initiation site stimulate RNAp III transcription. Transcription reactions, carried out as described in the Materials and Methods section, contained 40 μg of standard nuclear extract. Reactions contained 0.4 pmol of the downstream deletion templates pE2(+117) (lanes 1 and 3), and pE2(+62) (lanes 2 and 4), or of the templates pE2(Δ35/+117) (lanes 5 and 7) or pE2(Δ35/+62) (lane 6 and 8), from which sequences upstream of position −35 had also been deleted. The reactions whose products are shown in lanes 3, 4, 7 and 8 contained 2 μg/ml α-amanitin.

+120. As T1 ribonuclease is specific for guanosine residues, the protected products would be slightly longer than the actual transcripts. Two bands (t1 and t2), which corresponded to the sizes (59 and 104 nucleotides, respectively) expected for termination at the two aforementioned runs of T residues, were observed when the products of reactions containing the standard extract and 2 μg/ml α-amanitin were analyzed in this way (Figure 4, lane 2). No α-amanitin resistant transcripts extended beyond t2 (Figure 4). This fact was confirmed using a 3′ end-labelled S1 probe that spanned t2 (data not shown).

Several control reactions were performed in order to verify the assignment of the t1 and t2 bands. A deletion mutant, lacking sequences downstream of +62, generated the t1 band but not the t2 band (Figure 4, lane 4). An internal deletion mutant, Δ(91−100), missing the Ts of the t2 region (Figure 1) was transcribed using an RNAp II extract. When the resulting transcript is hybridized to the wild-type probe, a mismatch will occur to generate a protection product identical in length to t2 (104 nt) upon T1 ribonuclease digestion. As illustrated in Figure 4 (lane 11), a 104 nt protection product was indeed generated by RNAp II transcripts of the D(91−100) template. Moreover, RNA synthesized in the RNAp II extracts from a wild-type template or a deletion mutant (+62), yielded the protection products, of 124 nt and 66 nt respectively, expected for correctly initiated RNA reading-through the termination sites for RNAp III (Figure 4, lanes 9 and 12).

## Polymerase III transcription requires the same upstream promoter sequences as polymerase II transcription

As RNAp III was able to initiate transcription from the E2E promoter at the site used by RNAp II, we wished to determine whether the upstream elements required by RNAp II also governed the efficiency of initiation in the RNAp III system. The linker substitution mutations previously used in the RNAp II dependent system were, therefore, tested in the RNAp III dependent system. Transcription (in the presence of 2 μg/ml α-amanitin) was again found to be strongly dependent on the TATA-like sequence (Figure 5A, compare lanes 1 and 2), whereas a modest reduction (2-fold) in efficiency was found when either the ATF site or the distal E2F site were replaced with a linker (Figure 5B, lanes 10−12 and 7−9, respectively). Replacement of the proximal E2F site resulted in the stimulation of transcription (Figure 5B, lanes 4−6). It was previously shown that a distinct protein, Cα, binds to this proximal E2F binding site and was suggested that this protein may act as a transcriptional repressor (50). Our data are consistent with this model. As initiation of transcription from the E2E promoter by either RNAp II or RNAp III responded to the same upstream elements, analysis of upstream sequence elements alone provided no insight into the question of polymerase selectivity.

## An element downstream of the E2E increases the efficiency of polymerase III transcription

The 'classical' RNAp III genes have been characterized by intragenic promoters (1). The other group of RNAp III genes, which include upstream promoter elements, have been shown either to function without downstream promoter sequences (U6 or 7SK), or to require both motifs (7SL and EBER) (27−33).

We therefore made a series of downstream deletion mutations, and examined their effects on RNAp III transcription from the E2E promoter. The efficiency of E2E transcription was not altered when sequences downstream of position +117 were deleted (data not shown). If, however, sequences between positions +117 and +62 were removed, the efficiency of transcription was reduced approximately 2–3-fold (Figure 6, lanes 1–4). When sequences upstream of position −35 bp were deleted, transcription efficiency was similarly reduced 2–3-fold (Figure 6, lanes 5 and 7). This result is in accord with those shown in Figure 5B, which demonstrate that the ATF and the distal E2F binding sites increase the efficiency of transcription. If downstream sequences (+117 to +62) were removed from a template already lacking sequences upstream of −35, no further reduction in the level of transcription was observed (Figure 6, lanes 7 and 8). Conversely, sequences upstream of −35 bp were no longer found to stimulate transcription in the context of a promoter lacking sequences downstream of +62 bp (Figure 6, lanes 2, 6 or 4, 8). These properties suggest that elements upstream of position −35 and the downstream element(s) located between positions +62 and +117 may interact.

### A subpopulation of TFIID complexes is required for E2E transcription by RNA polymerase III

The chromatographic fractionation of whole cell or nuclear extracts on phosphocellulose yields four well defined fractions (A,B,C, and D), corresponding to an increasing affinity for the resin (34,35). Originally, the 'D' fraction was operationally defined as containing the TFIID factor (34), but it has recently been shown that more than one complex of this general transcription factor is present in HeLa whole cell extracts: a previously uncharacterized TFIID complex obtained from the phosphocellulose 'B' fraction ('B-IID') could replace the 'D' ('D-IID') fraction when reconstituting activity on the adenovirus major late promoter (36). Our initial attempts to reconstitute RNAp III dependent transcription from the E2E promoter using various combinations of phosphocellulose fractions showed no requirement for the phosphocellulose 'D' fraction (data not shown). We therefore chose to examine the TFIID requirement for the RNAp III dependent initiation of transcription on this promoter. Using a monoclonal antibody specific for TBP, we were able to partially deplete nuclear extracts of TFIID. Estimates based on immunoblots like that shown in Figure 7A indicated that between 60% and 70% of the TFIID in the nuclear extracts was removed. Concomitant with such removal of TFIID was a significant reduction (4-fold) in the efficiency of RNAp III transcription (Figure 7C, lanes 1,2 and 5). Increasing the amount of antibody by 2.5-fold had a minimal effect on the amount of TFIID removed from the extract (Figure 7A) or on the level of transcription (Figure 7C, lanes 2 and 5), suggesting that there is a population of TFIID that cannot be recognized by this antibody. Specific E2E transcription could be restored by the addition of the phosphocellulose 'B' fraction, but not by the addition of the phosphocellulose 'D' fraction (Figure 7C, compare lanes 3, 6 and 7 to lanes 4, 8 and 9). The concentrations of TFIID in the phosphocellulose 'B' and 'D' fractions, as measured by an immunoblot were almost equivalent (Figure 7B). Moreover the 'D' fraction contained active TFIID as measured by its ability to restore MLP transcription by RNAp II in a heat treated nuclear extract (37) (data not shown). In order to confirm that the phosphocellulose 'B' fraction was providing TFIID, and not stimulating transcription by providing a different limiting factor, we immunodepleted this fraction, and found that its ability to restore activity was then lost (Figure 7D, compare lanes 3 and 4 to lanes 5 and 6). The material bound to the antibody column during immunodepletion of the 'B' fraction was eluted with 2 M urea and dialyzed. When this fraction was added to the immunodepleted nuclear extract, transcriptional activity could be restored (Figure 7D, lanes 7 and 8). The concentration of TFIID in the 2 M urea eluate was approximately half of that of the phosphocellulose 0.3 M fraction (data not shown) and hence twice the volume was needed to restore a similar level of activity (Figure 7D).

## DISCUSSION

The E2E promoter of adenovirus type 2 includes a non-consensus TATA box as well as binding sites for the transcription factors E2F and ATF (Figure 1). In high salt nuclear extracts E2E transcription is almost exclusively carried out by RNAp II (Figure 2), in accord with results previously obtained with S100 extracts augmented with exogenous RNAp II (23,17). On the contrary, when moderate salt concentrations are used to extract the nuclei, and the extracts are not concentrated by ammonium sulfate precipitation, transcription is initiated by RNAp III. This conclusion is based on the insensitivity of E2E transcription in this system to low, but not high, concentrations of α-amanitin (Figure 3), as well as the finding that transcripts made in these extracts are terminated at two stretches of Ts (Figure 4). The E2E promoter can function to initiate RNAp III transcription with only a TATA element upstream (Figure 6). The presence of the RNAp II activator sequences that bind ATF or E2F results in modest (2–3-fold) increases in the efficiency of transcription by RNAp III (Figure 5B). Several examples of RNAp II activator sequences that stimulate RNAp III transcription have been observed among the class III genes that structurally resemble class II genes. In addition to ATF, which can also stimulate transcription of the 7S L (38) and the Epstein-Barr virus EBER genes (34), Oct-1 has been shown to stimulate U6 transcription (29) and SP-1 to activate H1 and MRP (39,40). Thus far, TFIID is the only general transcription factor that has been shown to be common to RNAp II and RNAp III transcription. The finding that several activating factors are able to stimulate transcription by both polymerases is consistent with the notion that many transcriptional activators perform by interacting with TFIID (41).

The ability of both RNAp II and RNAp III to initiate transcription at the same site in the E2E promoter is strikingly similar to the situation previously reported for the c-*myc* P1 and P2 promoters (14). In these cases, as with the E2E promoter, RNAp II or RNAp III transcription was dependent upon the particular extract used for transcription. Moreover, in oocytes, RNAp II transcripts were observed at low template concentrations, while RNAp III transcription required higher concentrations (15). A simple interpretation of all these observations is that RNAp III transcription can occur only when RNAp II, or a factor specifying RNAp II transcription, is limiting. As it is not known which polymerase might be limiting at a particular time and location *in vivo*, no inference can be made about the likelihood of whether RNAp III is used in the cell. However, the results of preliminary experiments indicate that small E2E RNA species are synthesized by RNAp III in nuclei isolated from adenovirus-infected cells (W. Huang, R.P. and S.J.F., unpublished observations). The similarity between the two promoters might suggest a role for RNAp III in attenuation

of transcription, a phenomenon that has been described for c-*myc* (42).

There is a notable sequence homology downstream of the initiation sites of the E2E and human c-*myc* promoters, between region +71 to +79 of the E2E and that of +30 to +38 of the c-*myc* sequence (Figure 1). This perfect match of 9 consecutive base pairs would occur randomly with a probability of less than one in two thousand, if one allows the freedom to slide 100 bp in search of such a match. This region is then a likely candidate for the E2E downstream stimulatory region that we have located between bp +62 and +117 (Figure 6).

Until quite recently there appeared to be a clear distinction between the structure of class II and class III genes (see Introduction). Class III genes were characterized by downstream

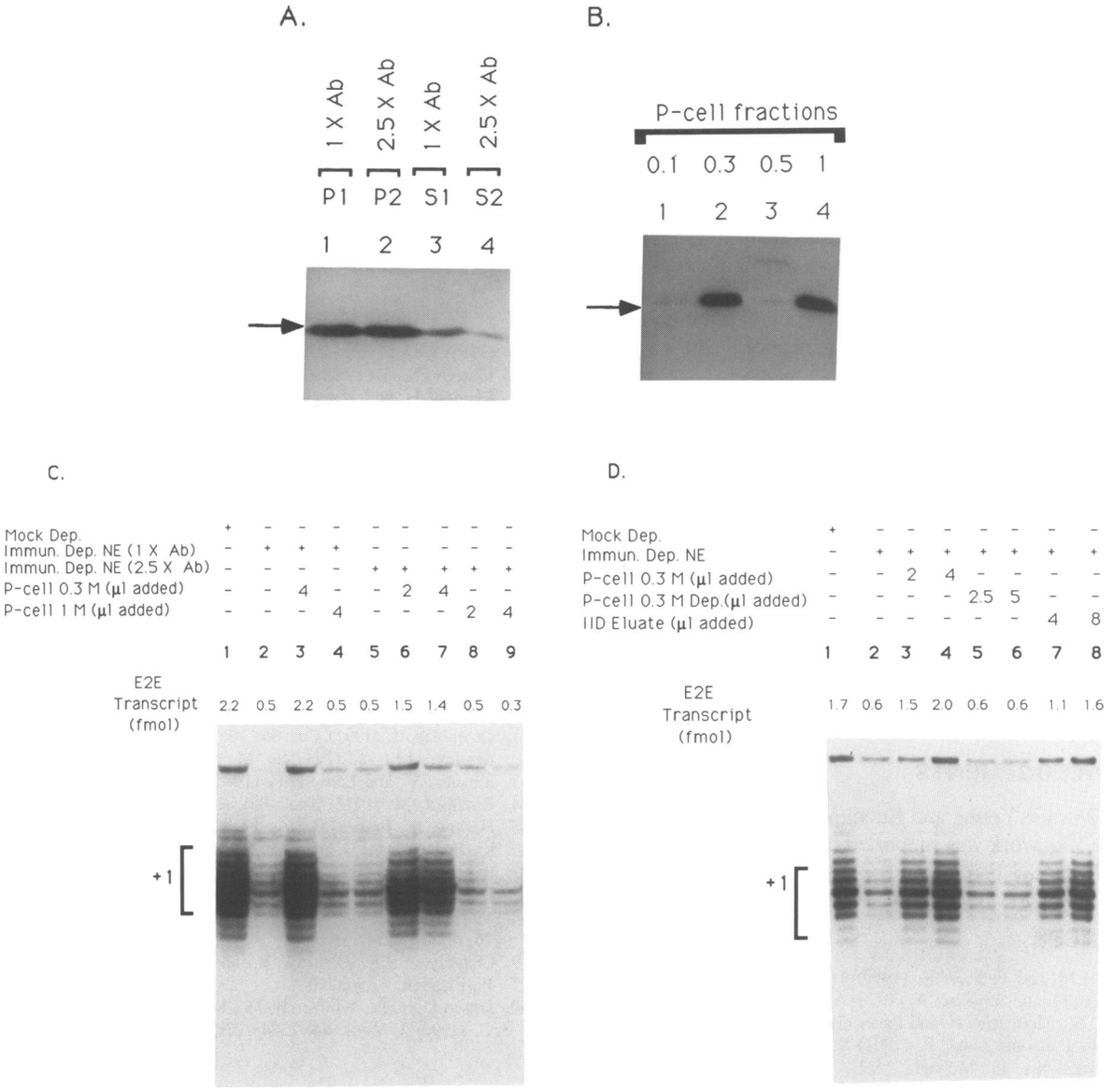


**Figure 7.** RNA polymerase III requires a specific subpopulation of TFIID to initiate transcription from E2E promoter. **A.** Immunoblotting with the anti-TBP monoclonal antibody described in the Materials and Methods section was used to compare the concentrations of TFIID in the pellets (P) and supernatants (S) recovered following immunodepletion of standard nuclear extract (110 µl, 5.5 mg protein/ml) The total pellets obtained by immunodepletion with 1.6 µg and 4 µg antibody, P1 and P2 respectively, were boiled in SDS sample buffer and compared to one half the respective supernatants, S1 and S2. **B.** Phosphocellulose fractions (50 µl) derived from nuclear extracts were separated by SDS-polyacrylamide gels, blotted onto nitrocellulose membranes, and probed with anti-TBP monoclonal antibodies. The arrow indicates the position of TBP. **C.** 110 µl of standard nuclear extract (5.5 mg protein/ml) was immunodepleted with either 4 µg M73 antibody (lane 1), 1.6 µg anti-TBP antibody (lanes 2–4), or 4 µg anti-TBP antibody (lanes 5–9), as described in the Materials and Methods section. Transcription reactions contained 44 µg of immunodepleted (or mock-depleted) extract, 0.4 pmol of pE2 template, and 2 µl (lane 6) or 4 µl (lanes 3 and 7) of 0.3 M phosphocellulose eluate (3.4 mg protein/ml) or 2 µl (lane 8), or 4 µl (lanes 4 and 9), or 1M phosphocellulose eluate (0.8 mg protein/ml). All reactions were carried out in the presence of 2 µg/ml of α-amanitin. **D.** Immunodepletions were carried out in the manner described in the legend to panel C, except that 120 µl of nuclear extract and 2 µg anti-TBP antibody were used. The 0.3 M eluate (3 ml) was depleted of TFIID by repeated passage (5×) over an anti-TBP column (0.6 ml, 2 mg anti-TBP antibody/ml resin). Transcription reactions contained mock depleted extract (lane 1), or immunodepleted extract (lanes 2–8), and 2 or 4 µl of the 0.3 M phosphocellulose eluate (3.4 mg protein/ml) (lanes 3 and 4), 2.5 and 5 µl of the TFIID-depleted 0.3 M phosphocelulose eluate (2.4 mg protein/ml), (lanes 5 and 6) or 4 and 8 µl of the 2 M urea eluate (0.8 mg protein/ml) from the anti-TBP column dialyzed into buffer A containing 0.1 M KCl (lanes 7 and 8).

promoter elements, which serve as binding sites for the RNAp III specific factors TFIIIC and/or TFIIIA. TFIIIC is believed to recruit the factor TFIIIB, which in turn specifies binding of RNAp III. Several RNAp III promoters that contain TATA elements at positions typical of class II genes, as well as upstream activating sequences typical of class II genes have now been identified. Some of these promoters (U6, 7SK) do not require downstream sequences for transcription *in vitro*, and U6 can be synthesized without TFIIIC (10). As these RNAp III genes have promoters that closely resemble those recognized by RNAp II, the mechanism by which the two polymerases are discriminated is not clear. One explanation proposed for polymerase selection is that the spacing between the TATA element and the proximal upstream factor binding site distinguishes the two classes of promoters (9). While this may be a necessary condition, it cannot be sufficient to explain the results described in this paper, as well as those previously reported for the c-*myc* gene (14): these data indicate that, for the same promoter structure, selectivity or exclusion of a particular polymerase is a function of the concentration of certain factors added. Only by using a reconstituted system with purified factors will it be possible to ascertain the critical step leading to formation of active RNAp II or RNAp III initiation complex.

Initiation of E2E transcription by RNAp III requires a form of TFIID found in the phosphocellulose 'B' fraction (Figure 7). Moreover, the ability to support RNAp III transcription from the E2E promoter copurified with TFIID during immunoaffinity chromatography (Figure 7). These results therefore suggest that in the case of the E2E promoter the particular subtype of TFIID makes a contribution to RNA polymerase selectivity, perhaps by determining whether TFIIB or TFIIIB is recruited to the promoter. Use of a promoter such as that of the E2E gene described here, or the c-*myc* promoter, which can be recognized by both polymerases, should facilitate identification of the factors that are required to select for either RNAp II or RNAp III.

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