The human adenovirus type 2 E2 early (E2E) transcriptional control region contains an efficient RNA polymerase II promoter, in addition to the well-characterized promoter for RNA polymerase III. To determine whether this promoter includes intragenic sequences, we examined the effects of precise substitutions introduced between positions +2 and +62 on E2E transcription in an RNA polymerase III-specific, in vitro system. Two noncontiguous sequences within this region were necessary for efficient or accurate transcription by this enzyme. The sequence and properties of the functional element proximal to the sites of initiation identified it as an A box. Although a B box sequence could not be unambiguously located, substitutions between positions +42 and +62 that severely impaired transcription also inhibited binding of the human general initiation protein TFIIIC. Thus, this region of the RNA polymerase III E2E promoter contains a B box sequence. We also identified previously unrecognized intragenic sequences of the E2E RNA polymerase II promoter. In conjunction with our previous observations, these data establish that RNA polymerase II and RNA polymerase III promoter sequences are superimposed from approximately positions -30 to +20 of the complex E2E transcriptional control region. The alterations in transcription induced by certain mutations suggest that components of the RNA polymerase II and RNA polymerase III transcriptional machines compete for access to overlapping binding sites in the E2E template.

Human subgroup C adenoviral proteins are synthesized in a strict temporal sequence during productive infection as a result of sequential activation of viral RNA polymerase II transcription units (see Refs. 1 and 2). The viral promoters recognized by components of the cellular RNA polymerase II machinery are analogous to those of cellular genes, indeed served as important models in early studies of RNA polymerase II transcription, but their activity is regulated by virus-specific mechanisms. The viral E1A proteins induce efficient transcription from early promoters by altering the activity or availability of a variety of cellular, sequence-specific transcriptional regulators or general initiation proteins (see Refs. 3, 4). Expression of viral late genes requires viral DNA synthesis in the infected cell and sequence-specific, transcriptional activators, including the IVa2 protein (5). Adenoviruses also depend on cellular RNA polymerase III (responsible for synthesis of tRNA, 5 S rRNA, and other small, host cell RNA species (see Refs. 6–8)), to transcribe the viral VA RNA I and VA RNA II genes. The promoters of these genes comprise two intragenic sequences closely related to similarly located promoter sequences of cellular genes, indeed served as important models in early studies of RNA polymerase III transcriptional control region (19, 20).

Although the adenoviral promoters recognized by the cellular RNA polymerase II and RNA polymerase III machines are generally typical of those of cellular genes, the E2 early (E2E) transcriptional control region is unusual. The E2 RNA polymerase II transcription unit, which encodes the viral replication polyprotein, is expressed from a well-characterized promoter (see Fig. 1) that contains a TATA-like sequence, two inverted binding sites for E2F, and an ATF recognition site (3, 4). Each of these sequences is required for both basal transcription, and E1A protein-mediated stimulation of transcription in infected cells (21, 22). However, RNA polymerase III also transcribes the 5′-end of this transcription unit both in vitro and in infected cells (23, 24) to produce small RNA species of some 45 and 90 nucleotides (see Fig. 1). Efficient E2E transcription by RNA polymerase III requires sequences 5′ to the site of initiation, notably the TATA-like sequence (23).2 Thus, promoters directing transcription by these two cellular enzymes are at least partially superimposed. An analogous arrangement of RNA polymerase III and II transcription units had been observed previously at the P1 and P2 promoters of the human c-myc gene (25–27). These RNA polymerase III promoters support efficient transcription in vitro, but c-myc mRNA synthesis by this enzyme is much less efficient than that by RNA polymerase II in Xenopus oocytes microinjected with the gene, and cannot be detected from the endogenous human genes of HeLa or HL60 cells (25, 27, 28). Thus, the adenoviral E2E transcriptional control region was the first documented to support transcription by both these cellular RNA polymerases in a normal biological context. Also atypical is the low concentration attained by each small E2E RNA made by RNA polymerase III: 10–20 copies per infected cell (24). This unusual property explains why E2E RNA polymerase III transcripts were not detected in the many

1 The abbreviations used are: E2E, E2 early; Ad2, adenovirus 2; PCR, polymerase chain reaction; bp, base pair(s).

2 W. Huang and S. J. Flint, unpublished observations.
previous studies of adenoviral gene expression. It also suggests that these RNAs are not likely to function in the same manner as the typical, abundant RNAs synthesized by RNA polymerase III. As a necessary prelude to genetic analyses of the function of the unorthodox adenoviral E2E RNA polymerase III transcription unit and its small RNA products, we are defining the sequences that comprise the RNA polymerase III promoter and their relationship to those of the overlapping (and essential) RNA polymerase II promoter. Here we report the results of mutational and biochemical analyses of intragenic sequences common to the E2E transcription units of RNA polymerases II and III.

**Experimental Procedures**

Templates for in Vitro Transcription and Their Mutagenesis—The wild-type E2E template for in vitro transcription was the plasmid pEI1, which contains the human adenovirus type 2 (Ad2) E2E sequence from position -249 to position +200. This plasmid was constructed by first inserting a NruI fragment comprising bp 26,893 to 27,187 of the viral genome into the ClaI site of the polylinker of pSP73 (Promega). A DNA fragment corresponding to positions 27,105 to 27,341 in the Ad2 genome, generated by PCR, was then introduced via BshHI and XhoI sites within the E2E RNA fragment and the pSP73 polylinker, respectively. The pEI1 t1 template, which was used as the parent in mutational analyses of intragenic promoter sequences (see “Results”), contained two T to A substitutions at positions +48 and +49 within the RNA polymerase III t1 termination site (see Fig. 1). Precise substitutions were introduced into the pEI1 t1 template by the unique site elimination method (29). A control “maxigene” template, to serve as an internal control for in vitro transcription reactions, was constructed by ligating a 30-bp random DNA sequence flanked by PvuII sites into the PvuII site (+62) of pEI1. A second series of templates, comprising the E2E DNA sequence from position -249 to position +62, or specifically mutated derivatives thereof, linked to the Photinus pyralis luciferase coding sequence was constructed for analysis of E2E transcription in whole cell extracts prepared from Ad2-infected cells. A DNA fragment encompassing the first 535 bp of the P. pyralis luciferase gene was amplified from P. pyralis PvuI1II fragments, or by the unique site elimination method. The PvuII/BglII-digested DNA was then ligated into similarly digested pEI1 t1 DNA to place the E2E promoter region from -249 to +62 immediately upstream of the luciferase sequence. The substitution mutations were created in the pEI1 t1 background were then introduced by swapping XhoI to PvuII fragments, or by the unique site elimination method. The nucleotide sequences of all pEI1 t1 and t1/Luc templates were confirmed by the chain termination DNA sequencing method (30).

Plasmid DNA templates for in vitro transcription reactions were purified using the Qiagen Maxi-prep protocol. The concentrations of the different templates to be compared, as well as their quality (i.e. presence of largely supercoiled DNA), were confirmed by ethidium bromide staining of DNA, following electrophoresis in 1.4% agarose gels, cast, and run in 0.4% Tris acetate, pH 8.0, containing 1 mM EDTA.

**In Vitro Transcription by RNA Polymerase III—**Transcription of E2E templates by RNA polymerase III was analyzed using nuclear extracts of HeLa cells prepared according to the procedure of Dignam et al. (31), in which at least 90% of the E2E transcripts are synthesized by RNA polymerase III (23). All transcription reactions contained 2 µg/ml a-amanitin to inhibit RNA polymerase II, 0.1 pmol of template DNA, 0.05 µmol of the internal control template described in the previous section, 1.33 mg/ml nuclear extract protein, 25 mM Hepes-KOH (pH 7.9), 30 mM KCl, 6 mM MgCl2, 600 µM each GTP, ATP, UTP, and CTP, 12% glycerol, and 2 mM dithiothreitol. Transcripts synthesized in 60 min at 30 °C were purified and analyzed by primer extension, as described previously (32, 33), using a saturating quantity (20 fmol) of a DNA primer complementary to positions +68 to +89 of the E2E transcription unit. The concentrations of the primer extension products of E2E and internal control transcripts were determined using a Molecular Dynamics PhosphorImager. Initial analyses of transcription from the E2E and pEI1 t1 templates employed an RNase T1 protection assay with an E2E riboprobe specifically end-labeled at positions +2, as described previously (24).

**In Vitro Transcription by RNA Polymerase II—**Transcription of wild-type and mutant E2E templates by RNA polymerase II was examined using whole cell extracts prepared as described previously (34) from HeLa cells infected with 15 plaque-forming units/cell Ad2 for 12 h, or from uninfected cells. Transcription reactions contained the quantities of template DNA listed in the figure legends, 0.1 pmol of the major late G-less cassette template, pML(CAT) (35) as an RNA polymerase II-specific internal control, 5.5 mg/ml Ad2-infected or uninfected cell extract protein, 67 mM KCl, 6.7 mM MgCl2, and other components as described above for RNA polymerase III transcription. The reactions also contained 40 µM tagetitoxin, a concentration empirically determined to inhibit both E2E and VA RNA I transcription by RNA polymerase III (see “Results”). Transcripts synthesized in vitro were analyzed by reverse transcription from the primer described above or a primer complementary to the luciferase sequence corresponding to positions +94 to +113 of the pEI1 t1/Luc template, which was used as the parent in mutational analyses of intragenic promoter sequences. The reactions were analyzed by primer extension, which allows both transcription of the wild-type E2E template produced approx-
Features of the Ad2 E2E transcription units. The region of the human Ad2 genome spanning the 5′-end of the RNA polymerase II transcription unit is depicted by the horizontal line at the top, on which features of the RNA polymerase II and III transcription units and promoters are indicated. The major site of initiation by RNA polymerase II, which is denoted by the jointed arrow, is defined as +1. The RNA polymerase III termination sites t1 and t2, each of which comprise 4 or more TA base pairs flanked by GC-rich sequences (23), are denoted by the inserted triangles. The vertical arrowhead at +68 represents the 5′-splice site between exon 1 and intron 1 of E2E pre-mRNA. The pre-mRNA and mRNA products of RNA polymerase II transcription and the small RNA species (E2E RNA I and RNA II) transcribed by RNA polymerase III are represented below.

Comparison of the Sites of Initiation of E2E Transcription by RNA Polymerses II and III—To map accurately the site(s) at which RNA polymerase III initiates E2E transcription, located previously in the vicinity of the major initiation site recognized by RNA polymerase II (23), primer extension was used to compare the 5′-ends of transcripts synthesized from the E2EΔt1 template in reactions in which only one of the two enzymes was active. The fungal toxin α-amanitin (at a low concentration, 2 μg/ml) and the bacterial phytotoxin tagetitoxin (40 μM) were exploited to inhibit RNA polymerases II and III, respectively. The latter compound has been reported to be a specific inhibitor of RNA polymerase III transcription from the type I and type 3 promoters of the 5 S RNA and U6 genes, respectively (38). It inhibited completely RNA polymerase III transcription from both the Ad2 VA RNA I and E2E promoters by a concentration of 30 μM, with no effect on RNA polymerase II transcription from the adenoviral major late promoter (data not shown). When RNA polymerase III was inhibited by the addition of tagetitoxin to the reaction, initiation was observed at a single site (Fig. 2B, lane 3) corresponding to the major cap site of E2E mRNAs made by RNA polymerase II in infected cells (39), by definition position +1. In contrast, RNA polymerase III transcription of the E2E template, initiated predominantly from position +2, although transcripts beginning at position −1 were also detected (Fig. 2B, lane 4). Thus, the two enzymes initiated transcription from distinct sites.

Identification of Intragenic Elements of the E2E RNA Polymerase III Promoter—Previous studies established that E2E sequences 3′ to position +62 are not necessary for efficient RNA polymerase III promoter activity in vitro (23), whereas deletion of sequences downstream of position +2 or of positions +22 to +62 reduced transcription to undetectable levels (data not shown). We therefore examined the effects of precise substitutions within the region +2 to +62 on the efficiency and accuracy of transcription. Both the 10- and 5-bp substitutions shown in Fig. 3, which were named according to the positions of the mutated base pairs, were introduced into the Δt1 background, as described under “Experimental Procedures.” The mutant and wild-type (Δt1) templates were transcribed in vitro by RNA polymerase III, and the products were analyzed by primer extension. To allow accurate quantification of the effects of the mutations on the efficiency of transcription, an E2E′ maxigene, which contains an insertion of a 30-bp random DNA sequence at position +62, was included in all transcription reactions. Typical results of these analyses are shown in Fig. 4, A and B, and the effects of the mutations upon the efficiency of RNA polymerase III transcription are summarized in Fig. 4C. None of the substitutions introduced immediately downstream of the initiation sites, such as mut2–6, mut7–11, or mut7–16 (Fig. 4A, lanes 3 and 4; Fig. 4B, lanes 3–5), or between positions +27 and +42 (Fig. 4A, lanes 3 and 7; Fig. 4B, lanes 3 and 10) resulted in significant inhibition of RNA polymerase III transcription (Fig. 4C). In contrast, the quantities of E2E transcripts detected in reactions containing the mut22–31, mut42–51, and mut52–62 templates were severely reduced, to values of 0.22, 0.04, and 0.06, respectively, relative to the wild-type concentration set at 1.00 (Fig. 4A, lanes 6, 8, and 9; Fig. 4C). None of these changes in the transcribed sequence altered the stability of the RNA made in the in vitro transcription reactions (data not shown), indicating that these substitutions inhibited transcription. Various 5-bp substitutions made between positions +42 and +62 also severely inhibited transcription, as did the mut48–57 substitution, which overlapped the 10-bp alterations initially introduced and analyzed (Fig. 4B, compare lanes 3, 11, 12, and 13; Fig. 4C). These properties suggest that the 21-bp segment occupying positions +42 and +62 comprises a single, functional promoter element. The data reported in Fig. 4, in conjunction with previous analysis of E2E templates carrying 3′-deletions (23), indicate that its 5′-boundary is close to position +62, whereas its 5′-boundary can be placed between positions +42 and +47. Mutation of the sequence +22 to +26 also resulted in inhibition of transcription (Fig. 4B, lane 10; Fig. 4C), confirming the presence of a second intragenic promoter sequence. In contrast, templates carrying mutations between positions +12 and +21 directed somewhat more efficient transcription by RNA polymerase III, with the greatest increase (a factor of 2.5) observed when the sequence between positions +14 and +19 was altered (Fig. 4B, lane 6; Fig. 4C).

These results indicated that the E2E RNA polymerase III promoter includes two blocks of internal sequence, occupying positions +22 to +26 and positions +42 to +62 of the transcription unit. This organization resembles that of type 2 RNA polymerase III promoters (see the introduction) as well as some that comprise both intragenic and essential upstream sequences, such as the promoter of the EBV EBER RNA 2 gene (40, 41). We therefore searched the intragenic E2E RNA polymerase III promoter sequences for consensus A and B box sequences (6). As illustrated in Fig. 5, the functionally defined promoter sequence +22 to +26 is included within a sequence that resembles the A box consensus sequence of tRNA genes: both the inhibitory substitutions mut22–26 and mut22–31 (Fig. 4C) reduced the match to the A box consensus sequence from 7/12 (Fig. 5) to 4/12 (Fig. 3) positions. The alterations in initiation specificity to include the +1 and +3 sites induced by all substitutions within this intragenic E2E sequence (Fig. 4A, lanes 4 and 5; Fig. 4B, lanes 5–8) provide strong support for its
The sequences altered in the mutant templates. The sequence of the surrogate wild-type E2E template was identical to the wild-type sequence except for point mutations in the t1 termination site shown in *boldface* and *italics*. The A box sequence is *highlighted* in gray. Positions that match the consensus A box sequence in Fig. 5 are shown in *boldface*. The sequences altered in the mutant templates listed at the *right* are shown below, with *boldface* indicating changes that maintain a match to the A box consensus.

**Fig. 3. Sequences of mutated E2E templates.** The sequence of the surrogate wild-type E2E template from positions +1 to +68 is shown at the *top*, with the mutation in the t1 termination site shown in *boldface* and *italics*. The A box sequence is *highlighted* in gray. Positions that match the consensus A box sequence in Fig. 5 are shown in *boldface*. The sequences altered in the mutant templates listed at the *right* are shown below, with *boldface* indicating changes that maintain a match to the A box consensus.

Classification as an A box, for such change in initiation specificity is characteristic of mutations within the A box sequence of type 2 RNA polymerase III promoters (*e.g.* Refs. 42–44). Furthermore, as described in the next section, substitutions within the A box consensus sequence shown in Fig. 5 impaired binding of human TFIIIC to the E2E promoter. Although templates carrying the mut14–19 and mut17–21 substitutions that alter the 5′ portion of the A box consensus sequence were transcribed more efficiently than the wild-type E2E template (Fig. 4C), these mutations did not improve the match to the consensus. Rather, both these substitutions reduced the match, to 6/12 positions, and each eliminated one of the two invariant base pairs of the consensus sequence, both of which are represented in the wild-type E2E sequence (Figs. 3 and 5). An alternative explanation for these observations is discussed below. In contrast to the ready identification of an A box consensus sequence, it was not possible to locate a B box by inspection of the more downstream E2E sequence required for efficient RNA polymerase III transcription; this 21-bp segment contains two adjacent sequences that are weak matches to the B box consensus sequence (Fig. 5). Such ambiguity prompted us to evaluate more directly the question of whether the E2E RNA polymerase III promoter contains a B box sequence.

**Human TFIIIC Components Recognize the Internal RNA Polymerase III Promoter Sequences**—The A and B box sequences of type 2 RNA polymerase III promoters are recognized by components of TFIIIC (see the introduction). Thus, if the internal elements of the RNA polymerase III promoter described above included functional A and B sequences, TFIIIC should bind specifically to the E2E promoter. To test this prediction, the interactions of human TFIIIC with the internal sequences of the Ad2 VA RNA I and E2E RNA polymerase III promoters were compared using electrophoretic mobility shift assays (45, 46). The human TFIIIC used in these experiments was partially purified by a previously described procedure (36), but was not subjected to B-box DNA-affinity chromatography (10). The latter procedure separates the B box binding component, TFIIIC2, from a second component, TFIIIC1, which is required for RNA polymerase III transcription and interacts strongly with termination signals and weakly with A box sequences (10, 13–15). Because a B box sequence had not been unambiguously identified in the E2E promoter, it seemed prudent to avoid separation of the promoter-binding proteins of TFIIIC.

As expected, human TFIIIC bound specifically to a DNA fragment spanning the A and B box sequences of the VA RNA I gene; formation of a pair of protein-DNA complexes was inhibited by homologous unlabeled DNA but not by an oligonucleotide unrelated in sequence (Fig. 6A, *lanes* 1–3). The mobility of these two complexes relative to free DNA and to one another suggests that they represent previously described TFIIIC2-DNA complexes containing transcriptionally active...
and inactive forms of this initiation protein (12, 13). An E2E DNA fragment containing all sequences transcribed by RNA polymerase III also inhibited binding of TFIIIC to the internal promoter sequences of the VA RNA I gene (Fig. 6A, lanes 1 and 4). When this E2E DNA fragment was used as a probe, a set of specific protein-DNA complexes, two of which exhibited the same mobilities as the TFIIIC-VA I DNA complexes, was observed (Fig. 6A, lanes 5, 6, and 8). Their formation was inhibited by both homologous, unlabeled DNA and the DNA containing the VA RNA I promoter (Fig. 6A, lanes 5, 7, and 8). These results established that human TFIIIC specifically recognizes internal sequences of the E2E RNA polymerase III promoter.

We next examined the relationship between binding of TFIIIC to the internal E2E promoter sequence and the initiation of transcription by RNA polymerase III, by determining whether mutations that inhibited E2E transcription altered the binding of the protein. The abilities of wild-type DNA and mutant DNA fragments carrying specific substitutions to act as competitors of binding of TFIIIC to 32P-labeled E2E DNA were compared as a function of competitor concentration. The mut27–31 substitution, which did not decrease the efficiency of E2E transcription by RNA polymerase III (Fig. 4, B and C), did not impair binding of TFIIIC to wild-type E2E DNA (Fig. 6B, compare lanes 1–5 and 11-15). Mutations within the portion of the E2E sequence resembling an A box inhibited TFIIIC binding to the internal E2E promoter DNA (Fig. 5B, compare lanes 1, 2, and 5), but by only a modest degree (Table I). The correlation between the effects of the substitution mutations on E2E transcription by RNA polymerase III (Fig. 4) and on binding of TFIIIC to the internal E2E promoter DNA (Fig. 5B) established that human TFIIIC specifically recognizes internal sequences of the E2E RNA polymerase III promoter.

**FIG. 4.** Identification of intragenic sequences required for E2E transcription by RNA polymerase III. Transcription of the wild-type E2E template Δt1 or its mutated derivatives carrying 10-bp (A) or 5-bp (B) substitutions between positions +2 and +62 was carried out under the RNA polymerase III-specific conditions described under “Experimental Procedures.” Transcription reactions contained 0.05 pmol of the internal control E2E maxigene template and no test template (lane 2 in A and B) or 0.1 pmol of wild-type E2EΔt1 DNA (A and B, lane 3) or the mutant templates mut7–16, mut17–26, mut22–31, mut32–41, mut42–51, and mut 52–62 (A, lanes 4–9, respectively) or mut2–6, mut7–11, mut12–18, mut14–19, mut17–21, mut22–26, mut27–31, mut42–47, mut48–57, and mut58–62 (B, lanes 4–13, respectively), which are designated m7–16 and so on. Transcripts were detected by primer extension, followed by electrophoresis of cDNA products under denaturing conditions and autoradiography (see “Experimental Procedures”). The positions of the cDNAs synthesized from the E2E (E2E) and internal control (IC) templates are indicated at the right, and the lengths of DNA markers (5′-end-labeled HaeIII fragments of pBR322 DNA run in lane 1 in each of the gels shown) are listed at the left. The specific E2E cDNAs were quantified directly using a Molecular Dynamics PhosphorImager, and values obtained for each mutant template were corrected using those of products of transcription of the internal control template. In C, these corrected values are expressed relative to that obtained from the wild-type E2EΔt1 template, set at 1.0. The relative activity shown for each mutant template represents the mean of three independent experiments.

**FIG. 5.** Comparison of intragenic E2E RNA polymerase III promoter sequences to tRNA consensus A and B boxes. The sequence of the Ad2 E2E transcription units extending to position +68 is shown at the top. Sequences required for efficient or accurate transcription by RNA polymerase III are underlined, whereas those necessary for maximally efficient transcription by RNA polymerase II are indicated by the *line above* the sequence. Consensus A and B box sequences (6, 44) and typical distances between them are shown below, with invariant nucleotides in larger sized font, where R = a purine, Y = a pyrimidine, and N = any nucleotide. The best matches of E2E sequences within the functionally defined promoter regions are indicated *below*, with conserved nucleotides in *boldface*. 

![Diagram of Superimposed RNA Polymerase II and III E2E Promoter Sequences](image-url)
TABLE I

Relative affinities of TFIIC for mutated E2E promoter sequences

| E2E promoter | Relative affinity of TFIIC binding |
|--------------|-----------------------------------|
| Wild-type    | 1.00                              |
| mut14–19     | 0.45                              |
| mut22–26     | 0.39                              |
| mut27–31     | 1.00                              |
| mut42–47     | 0.21                              |
| mut48–57     | 0.18                              |
| mut56–62     | 0.12                              |

"Determined as described under "Experimental Procedures.""

TFIIC components to E2E DNA (Table I) indicated that binding of TFIIC to internal promoter sequences is required for RNA polymerase III transcription. Thus, the intragenic sequences required for E2E transcription by RNA polymerase III appear to be functionally equivalent to those of the well-characterized type 2 promoters recognized by this enzyme.

Identification of Internal Sequences Required for RNA Polymerase II Transcription—As the contribution of transcribed sequences to the E2E RNA polymerase II promoter had never been examined, we also determined the effects of the intragenic mutations on transcription by this enzyme. Initial surveys of E2E transcription by RNA polymerase II and RNA polymerase III, distinguished by their sensitivities to 2 μg/ml α-amanitin and 40 μM tagetitoxin, respectively, indicated that RNA polymerase II transcription was less efficient than that by RNA polymerase III, but that RNA polymerase III transcription invariably predominated in both nuclear and whole HeLa cell extracts (data not shown). However, two conditions that increased the efficiency of E2E transcription by RNA polymerase II relative to that by RNA polymerase III were identified. Under in vitro conditions typically used for RNA polymerase II transcription, the proportion of the total E2E RNA synthesized by this polymerase was 2- to 3-fold greater in whole HeLa cell extracts prepared from cells harvested 8–12 h after Ad2 infection than in uninfected cell extracts prepared in parallel (data not shown), reaching some 30% of the total (Fig. 2B). This difference could be the result of stimulation of RNA polymerase II transcription from the E2E promoter by the viral E1A proteins (see Ref. 4). We also observed that the fraction of E2E RNA made by RNA polymerase II in uninfected cell extracts increased to values like those supported by Ad2-infected cell extracts when the concentration of template DNA was reduced (data not shown). To avoid possible complications that might be introduced by effects of viral proteins, the latter condition was used to examine the effects of the E2E mutations described previously on RNA polymerase II transcription. A G-less cassette under the control of the viral ML promoter served as an internal control (see “Experimental Procedures”). Typical results of these experiments are shown in Figs. 7, A and B, and the quantification of several is summarized in Fig. 7C.

Substitution of sequences between positions +32 and +62 did not significantly alter the efficiency of RNA polymerase II transcription (Fig. 7B, compares lanes 1 and 5–8; Fig. 7C). However, mutation of the sequence adjacent to the initiation site (+2 to +11), as well as a more downstream segment (+14 to +21), resulted in inhibition of transcription by up to a factor of 8 (Fig. 7A, lanes 1–3 and 5–7; Fig. 7C). Thus, the E2E RNA polymerase II promoter extends for some 20 bp beyond the initiation site. Although substitution of positions +12 to +16 did not inhibit transcription (Figs. 7A, lanes 1 and 4), direct inhibitory effects on RNA polymerase II transcription might be offset by relief of competition from RNA polymerase III transcription (Fig. 3, “Discussion”), for this substitution impinges on the A box of the RNA polymerase III promoter (Fig. 5). Substitution of positions +27 to +31 increased the efficiency of RNA transcription, by some 2.5-fold (Figs. 7B, lanes 1 and 4, and 7C). None of these intragenic sequence mutations altered the specificity of initiation of E2E transcription by RNA polymerase II (Fig. 7, A and B), indicating that this parameter is determined by RNA polymerase II promoter sequences lying upstream of the initiation site.

DISCUSSION

The promoters of many cellular and viral genes transcribed by RNA polymerase III include intragenic A and B boxes (see Refs. 6, 8, 16 for reviews). The adenoviral E2E RNA polymerase III promoter also contains two intragenic sequences required for efficient or accurate transcription by this enzyme (Fig. 4). The sequence of the initiation site-proximal promoter element (Fig. 5) and the alterations in initiation specificity observed when it was mutated (Fig. 4, A and B) allow it unambiguous identification as an A box. The specific binding of human TFIIC to internal sequences of the E2E RNA polymerase III transcription unit (Fig. 6) confirms that this viral promoter contains a B box (10, 13–15). The observation that mutations within the sequence +42 to +62 strongly impaired both transcription (Fig. 4) and TFIIC binding (Fig. 6B; Table I) locate the E2E B box to within this 21-bp segment of the transcription unit. However, two nonoverlapping candidate sequences lie within this region (Fig. 5). Several arguments suggest that the E2E sequence located between positions +51 and +61 is the
binding site for TFIIIC2. This sequence includes three times as many of the invariant base pairs of the B box consensus sequences as the functionally defined sequence immediately 5′ to it (Fig. 5). Furthermore, a B box at positions +51 to +61 would yield a separation of E2E A and B box sequences of 26 bp, closer to the range typically observed (see Refs. 6, 8, 16, 47) and required for optimal type 2 promoter function (e.g., Refs. 42, 48).

Specific binding of the TFIIIC1 component of TFIIIC to the termination regions of the Ad2 VA RNA I gene and a Xenopus laevis rRNA 16S gene has been reported to stabilize binding of TFIIIC2 to the promoters to support maximally efficient transcription (14). The E2E t1 termination site for RNA polymerase III transcription (Fig. 1) is included within the 10-bp sequence lying 5′ to the putative B box. Thus, TFIIIC1 may bind to a region that includes this sequence to stabilize otherwise low affinity binding of TFIIIC2 to the adjacent B box. Further experiments will be required to determine whether such a mechanism of TFIIIC binding accounts for the requirement for an unusually long, initiation site-distal, intragenic sequence.

The E2E RNA polymerase III promoter does not belong to one of the three types now generally recognized (see Refs. 6, 8, 16), but rather closely resembles that of the EBV EBER RNA 2 gene: both include intragenic A and B boxes and upstream TATA-like sequences (Fig. 5 (23, 40)) and binding sites for sequence-specific activators of RNA polymerase II transcription that are necessary for maximal activity of the promoters (23, 40). Despite such similarities, the EBV EBER RNA 2 gene is transcribed only by RNA polymerase III. One important determinant of such exclusivity is its TATA-like sequence, designated ETAB, located at positions −25 to −30 (41). The E2E TATA-like sequence, TTAAGAGT, is noncanonical, for it includes GC base pairs at positions analogous to those of the ETAB sequence. However, in contrast to this latter sequence, the E2E TATA sequence is also part of a functional RNA polymerase II promoter (see the introduction and Fig. 1) and behaves as a typical RNA polymerase II TATA sequence in assays for TBP binding and transcriptional function. It will be of considerable interest to identify the features of the E2E TATA sequence that allow recognition by TBP-containing initiation proteins specific for both RNA polymerase II and RNA polymerase III.

Mutations introduced between positions +14 and +21, a sequence that includes most of the A box of the RNA polymerase III promoter (Fig. 5), significantly inhibited E2E transcription by RNA polymerase II (Fig. 7). Thus, a single intragenic E2E sequence functions as part of both the RNA polymerase II and the RNA polymerase III promoters (Fig. 5). This arrangement seems likely to account for the initially surprising observation that mutations introduced into the 5′-end of the A box altered initiation specificity but either did not reduce, or modestly increased, the efficiency of transcription by RNA polymerase III (Fig. 4). In the reactions specific for this enzyme, RNA polymerase II was inhibited by addition of an appropriate concentration of α-amanitin. This toxin binds to the largest subunit of RNA polymerase II and inhibits transcription after assembly of preinitiation complexes and formation of the first phosphodiester bond in nascent RNA (see Ref. 49). Consequently, when a mutation alters both the RNA polymerase III and the RNA polymerase II promoters, such as several that impinge on the A box, direct inhibition of RNA polymerase III transcription may be offset by inhibition of formation of RNA polymerase II initiation complexes. Thus, RNA polymerase III transcription would appear unchanged or stimulated, as indeed observed (Fig. 4). The properties of the E2E template with a substitution at the 3′-end of the A box (positions +22 to +26) provide additional support for the hypothesis that proteins mediating recognition of the RNA polymerase II and the RNA polymerase III promoters are in competition for superimposed binding sites: This mutation had no significant effect on the efficiency of transcription by RNA polymerase II (Fig. 7, B and C), and inhibited E2E transcription by RNA polymerase III by some 90% (Fig. 4, B and C).

Substitutions between positions +2 and +11 that inhibited RNA polymerase II transcription did not stimulate RNA polymerase III transcription (Figs. 4 and 7). Conversely, when the B box-containing region of the RNA polymerase III promoter was crippled, no increase in transcription was observed in the RNA polymerase II-specific system, in which tagetitoxin inhibits elongation by RNA polymerase III with no apparent effect on preinitiation complex assembly (50). It therefore appears that binding of TFIIIC to the RNA polymerase III promoter neither interferes directly with recognition of the RNA polymerase II promoter, nor is the reaction rate-limiting for assembly.

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3 H. Chen and S. J. Flint, unpublished observation.
of stable preinitiation complexes committed to transcription by RNA polymerase II under the conditions used in these experiments. A number of parameters, including absolute and relative concentrations of initiation proteins specific for each RNA polymerase, their affinities for binding sites in the template or in other proteins, and, in the cells, the relative concentrations of the RNA polymerase holoenzymes that are believed to mediate initiation of transcription (see Refs. 51–53), seem likely to govern which reactions will be in competition under different conditions. It will therefore be important to establish which sequences of the RNA polymerase II and RNA polymerase III promoters set the balance of E2E transcription by the two RNA polymerases in adenovirus-infected cells.

Because E2E RNA I contains only the 5′-half of the sequence of the longer E2E RNA II (Fig. 1), it is unlikely that these RNA species conform to a common structure necessary for a common function. In fact, the run of four T-A base pairs of the E2E t termination site of Ad2 or Ad5 is not conserved even in the genomes of all human members of the Mastadenoviridae, and elimination of the t site does not impair replication of subgroup C adenoviruses.4 These properties of the small E2E RNAs, and the low concentrations at which they are present in infected cells (24), suggest that production of functional RNA species may not be the primary purpose of transcription of E2E sequences by RNA polymerase III. Rather, such transcription might negatively regulate expression of the viral replication proteins encoded in the RNA polymerase II E2E transcription unit. The competition for access to superimposed sequences of the two E2E promoters discussed above is also consistent with such a regulatory role. Analogous function as regulators of the two E2E promoters discussed above is also consistent with unit. The competition for access to superimposed sequences of the RNA polymerase III promoters set the balance of E2E transcription by the two RNA polymerases in adenovirus-infected cells.

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