Unusual Properties of Adenovirus E2E Transcription by RNA Polymerase III

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In adenovirus type 5-infected cells, RNA polymerase III transcription of a gene superimposed on the 5′ end of the E2E RNA polymerase II transcription unit produces two small (<100-nucleotide) RNAs that accumulate to low steady-state concentrations (W. Huang, R. Pruzan, and S. J. Flint, Proc. Natl. Acad. Sci. USA 91:1265-1269, 1994). To gain a better understanding of the function of this RNA polymerase III transcription, we have examined the properties of the small E2E RNAs and E2E RNA polymerase III transcription in more detail. Although the RNA polymerase III transcripts were present at significantly lower concentrations than E2E mRNA throughout the period examined, E2E transcription by RNA polymerase III was found to be at least as efficient as that by RNA polymerase II. The short half-lives of the small E2E RNAs estimated by using the actinomycin D chase method appear to account for their limited accumulation. The transcription of E2E sequences by RNA polymerase II and III in cells infected by recombinant adenoviruses carrying ectopic E2E-CAT (chloramphenicol transferase) reporter genes with mutations in E2E promoter sequences was also examined. The results of these experiments indicate that recognition of the E2E promoter by the RNA polymerase II transcriptional machinery in infected cells limits transcription by RNA polymerase III, and vice versa. Such transcriptional competition and the properties of E2E RNAs made by RNA polymerase III suggest that the function of this viral RNA polymerase III transcription unit is unusual.

The double-stranded DNA genomes of subgroup C human adenoviruses such as adenovirus type 5 (Ad5) contain the coding sequences for over 40 proteins, organized into eight RNA polymerase II transcription units (47). Multiple mRNAs specifying different proteins are produced from the primary transcripts of all but two of these transcription units by alternative splicing and polyadenylation (18, 47). The densely packed adenoviral genome also contains genes transcribed by cellular RNA polymerase III, the enzyme responsible for the synthesis of small cellular RNAs that function in such processes as translation (iRNAs, 5S RNA), pre-mRNA splicing (U6 snRNA), and protein trafficking (7SL RNA) (23, 42, 57).

Two such RNA polymerase III transcription units, encoding virus-associated (VA) RNA I and VA RNA III, are located within an intron of the Ad5 major late RNA polymerase II transcription unit (37, 44). Although the number, size, and genomic location of VA RNA genes vary among the Adenoviridae, the majority of adenoviral genomes examined to date contain at least one such gene. Both VA RNAs synthesized in Ad2- or Ad5-infected cells are very abundant and accumulate in the cytoplasm (37). The major species, VA RNA I, is necessary for efficient translation of viral late mRNAs, as it blocks activation of RNA-dependent protein kinase, and hence inhibitory phosphorylation of eIF2-α, in infected cells (35, 37).

In initial studies of low-molecular-mass adenoviral RNAs, species other than the VA RNAs were not detected by methods with a lower sensitivity limit estimated to be on the order of 10³ RNA molecules/cell (36). However, an additional class III gene is present in the viral genome, superimposed on the 5′ end of the E2E RNA polymerase II transcription unit. In both in vitro transcription systems and Ad5-infected cells, RNA polymerase III synthesizes small E2E RNAs of some 45 and 90 nucleotides (28, 43). This enzyme initiates E2E transcription in the immediate vicinity of the major initiation site for RNA polymerase II (by definition, position +1), most frequently at position +2, but terminates transcription at two sites designated t1 (+45) and t2 (+90) (Fig. 1) (16, 28, 43). In contrast to the VA RNAs and many cellular RNAs made by RNA polymerase II, the small E2E RNAs, which have been called E2E RNA I and E2E RNA II (Fig. 1), accumulate to only very low concentrations (28). This property suggests that the E2E RNA polymerase III transcripts function in a manner previously unknown among viral RNAs synthesized by this enzyme.

The E2E RNA polymerase II promoter includes a TATA-like sequence and binding sites for the cellular transcriptional activators ATF and E2F located within the 90-bp sequence upstream of the initiation site (47, 48) (Fig. 1). Each of these sequences is required for stimulation of RNA polymerase II transcription by viral E1A proteins in infected cells (32, 53). Sequences located between positions +1 and +30 are also necessary for maximally efficient E2E transcription by RNA polymerase II in vitro (16). The results of mutational analysis of the organization of the E2E RNA polymerase III promoter indicate that it too comprises both internal and upstream sequences (16, 43). The internal sequences conform to those present in the majority of promoters recognized by RNA polymerase (23): the E2E promoter contains an A box with typical sequence and functional properties and a B box that binds the...
human RNA polymerase III initiation protein TFIIIC. The A box sequence partially overlaps an internal sequence of the RNA polymerase II promoter (16). Maximally efficient E2E transcription by RNA polymerase III also requires an upstream sequence located in the vicinity of the TATA box (16, 43). Sequences of the RNA polymerase II and RNA polymerase III promoters are therefore superimposed between positions −30 and +30 of the adenoviral E2E transcription units. Although unusual, this arrangement of transcription units and control sequences for RNA polymerases II and III is not unprecedented. An RNA polymerase III transcription unit has been reported to be superimposed on the 5′ end of the c-myc gene transcribed by RNA polymerase II (3, 11, 50). While the c-myc RNA polymerase III promoter is active in vitro systems and Xenopus oocytes, its transcripts have not been detected in mammalian cells in which c-myc mRNA is made (3, 4, 50). However, the 5′ transcriptional control region of the Xenopus class II gene encoding TFIIIA contains an RNA polymerase III transcription active in vivo (34).

The superimposition of RNA polymerase II and III promoters and transcription units in the adenoviral genome raises the issue of whether recognition of its E2E promoter by components of the RNA polymerase III system interferes with E2E transcription by RNA polymerase II. Such a phenomenon could influence significantly the course of the infectious cycle: E2E mRNAs encode all three of the viral replication proteins (47), and their production is therefore rate limiting for the onset of viral DNA and the transition into the late phase of infection. To assess the potential for such transcriptional interference and possible functions of E2E transcription by RNA polymerase III, we have examined the properties of both E2E transcription by RNA polymerase III and its products during the Ad5 infectious cycle.

**MATERIALS AND METHODS**

**Cells and virus.** HeLa cells were maintained in suspension culture in SMEM (Gibco-BRL) supplemented with 5% calf serum (Gibco-BRL). HeLa and 293 cells were grown in monolayers in Dulbecco’s modified Eagle’s medium (Gibco-BRL) containing 10% calf serum. Ad5 (wild-type 300) was propagated in HeLa cells in suspension culture, and the virus titers were determined by plaque assay on HeLa cells as described previously (20). Recombinant derivatives of Ad5 carrying ectopic, chimeric genes that comprise wild-type or mutated E2E promoters (positions −264 to +62) linked to a chloramphenicol acetyltransferase reporter gene (32) were amplified and assayed in the same way.

**Analysis of steady-state RNA concentrations.** HeLa cells in suspension culture were infected with 20 PFU of Ad5 per cell and harvested after increasing periods of infection or were mock infected. Cytoplasmic and nuclear fractions were separated, and RNA was purified from them as described previously (20, 28). Purified RNA samples were stored in portions of 20 to 50 μg as ethanol precipitates at −80°C. To detect simultaneously E2E mRNA (exon 1) and the E2E RNA species synthesized by RNA polymerase III, cytoplasmic RNAs were hybridized to a 32P-labeled, antisense riboprobe complementary to positions −16 to +120 of the E2E transcription unit and digested with RNase T1, as described previously (43). The products of digestion were separated by electrophoresis in 8% polyacrylamide gels (1:19 bisacrylamide) containing 8 M urea, 90 mM Tris, 80 mM sodium borate, and 2 mM EDTA and detected by autoradiography of dried gels. To establish when the late phase of infection began, the same RNA samples were assayed for the viral, late IVa2 mRNA by using a nuclease S1 protection assay as described previously (43) with a 5′-end-labeled oligonucleotide complementary to positions −15 to +45 of the IVa2 genes. Protection products were analyzed as described above.

Run-on transcription assays in nuclei isolated from HeLa cells infected with 20 PFU of Ad5/cell for increasing periods or from HeLa cells infected with 20 PFU of recombinant E2E-CAT viruses (see Fig. 6A), and to unrelated pUC19 DNA. In experiments examining the time course of E2E transcription by RNA polymerases II and III, the membranes also carried plasmids containing sequences of the viral major late and VA RNA 1 genes. Hybridization, washing, and RNase A treatment of membranes were carried out as described previously (24, 27). Hybridization signals were detected and quantified by using a Molecular Dynamics PhosphorImager or by direct scintillation counting of membrane segments following autoradiography.

**In vitro E2E transcription by RNA polymerase III.** The pEII template was transcribed in HeLa cell nuclear extracts (12) in the presence of 2 μg of α-amanitin per ml to inhibit RNA polymerase II, and the RNA was purified as described previously (43).
In Materials and Methods (Fig. 2, compare lanes 2 and 3 with 4). However, some differences in the populations of the E2E RNA polymerase III transcripts synthesized in vitro and in infected cells were observed. The infected cells’ cytoplasmic RNA population contained much smaller quantities of E2E RNAs 20 to 25 nucleotides longer than RNAs I and II than those present among the products of in vitro transcription reactions (Fig. 2, compare lanes 3 and 4). These E2E RNAs are of lengths predicted for initiation of RNA polymerase III transcription in the vicinity of a second, upstream site (−26) at which RNA polymerase II can initiate E2E transcription in vitro (38, 58). It has been reported that the +1 site is far and away the most frequent site of initiation of E2E transcription by RNA polymerase II in infected cells, accounting for some 95% of total transcription (52), as also observed here (Fig. 2, lanes 2 and 3). The data shown in Fig. 2 indicate that RNA polymerase III also initiates E2E transcription preferentially at this initiation site in Ad5-infected cells.

The concentration of E2E RNA II relative to that of E2E RNA I was also much lower in infected-cell RNA preparations than when E2E DNA was transcribed in vitro (Fig. 2, compare lanes 2 and 3 with lane 4). Both in vitro and in vivo, RNA polymerase III terminates E2E transcription at the two sites shown in Fig. 1, and in vitro it initiates transcription at positions −1 and +2, with a strong preference for the +2 site (16, 28, 43). The differences in the relative concentrations of E2E RNA I and RNA II in infected cells, as well as in the representation of the E2E RNA I species (Fig. 2, compare lanes 3 and 4), might therefore be the result of differences in the utilization of termination and initiation sites, respectively. However, posttranscriptional cleavage of the small E2E RNAs in infected cells (see below) might also contribute to the differences in the populations of these RNA species made in infected cells or in vitro reactions.

The cytoplasmic accumulation of the products of E2E transcription by RNA polymerase II and III during the infectious cycle was examined by using the RNase protection assay described above. Both E2E mRNA and the small RNAs made by RNA polymerase III could be detected at 4 h postinfection (p.i.), and both increased in steady-state concentration by 12 h p.i. (Fig. 3A, lanes 2 to 4). The same pattern of accumulation was observed when nuclear E2E RNAs were examined (data not shown). Under the conditions of infection used in these experiments, the viral late IVa2 mRNA was first detected at 8 h and increased substantially in concentration by 12 h p.i. (Fig. 3C, lanes 1 to 3). Thus, the production of the E2E RNA polymerase III transcripts did not depend on viral DNA synthesis in infected cells. Consistent with the classification of these transcripts as early RNAs, the inhibitor of viral DNA synthesis, cytosine arabinoside, reduced only modestly the accumulation of all three E2E RNAs (Fig. 3B, lanes 1 and 2), while blocking completely the synthesis of IVa2 mRNA (Fig. 3C, compare lanes 3 and 4).

The results of experiments like that shown in Fig. 3 indicated that the relative cytoplasmic concentrations of E2E RNAs synthesized by RNA polymerase II and III do not change significantly as the infectious cycle proceeds. We have previously estimated that, at 14 h after infection, each E2E RNA polymerase III transcript is present at a total concentration of <20 copies/infected cell (28). In contrast, the E2E mRNA

FIG. 2. Comparison of E2E RNA polymerase III transcripts synthesized in Ad5-infected cells and in vitro. Cytoplasmic RNA purified from mock-infected HeLa cells (32 μg, lane 5) or from cells infected with 20 PFU/cell for 12 h (lanes 2 and 3, 16 and 32 μg, respectively) and the products of E2E transcription by RNA polymerase III in vitro (lane 4) were analyzed by using an RNase T1 protection assay and electrophoresis in an 8% polyacrylamide sequencing gel, as described in Materials and Methods. The positions of products generated by exon 1 of E2E mRNA and E2E RNAs I and II are indicated by the black arrows on the right. The predicted length of each protection product is also listed. Less-than-full-length RNA I is indicated by the dashed, gray arrow at the right, while the barbed arrow heads show the positions of RNAs 20 to 25 nucleotides longer than E2E RNA I and RNA II (see text). The lengths of 32P-labeled DNA fragments used as markers (lane 1) are listed on the left.

Estimation of the stabilities of E2E RNA polymerase III transcripts. HeLa cells were infected with 20 PFU/cell for 12 h, and one portion of the culture was harvested. Actinomycin D was then added to the remainder to a final concentration of 10 μg/ml, and equal numbers of cells were collected after increasingly long periods of incubation in the presence of the drug. Cytoplasmic RNA was prepared and assayed for E2E RNAs by using the RNase T1 protection assay described above, with an antisense E2E RNA 3′ end labeled at position +2 (28).

RESULTS

Kinetics of production of E2E RNAs synthesized by RNA polymerase III. As a first step toward more-detailed characterization of E2E transcription by RNA polymerase III, we compared the kinetics of production of the small RNAs made by this enzyme with those of production of E2E mRNA during the initial period of the infectious cycle. An RNase T1 protection assay was used to detect, in a single reaction, E2E mRNA during the initial period of the infectious cycle. An RNase T1 protection assay was used to detect, in a single reaction, E2E mRNA synthesized by RNA polymerase II and III during the infectious cycle was examined by using the RNase protection assay described above. Both E2E mRNA and the small RNAs made by RNA polymerase III could be detected at 4 h postinfection (p.i.), and both increased in steady-state concentration by 12 h p.i. (Fig. 3A, lanes 2 to 4). The same pattern of accumulation was observed when nuclear E2E RNAs were examined (data not shown).
encoding the DNA-binding protein accumulates to several hundred copies per cell (21; S. J. Flint, unpublished observations). If the steady-state concentrations of the E2E RNAs were determined primarily by their rates of synthesis, these properties would imply that RNA polymerase II transcribed E2E sequences significantly more efficiently than did RNA polymerase III throughout the initial period of the infectious cycle. To test this prediction, the rates at which RNA polymerase II and III transcribe the common sequence that comprises the 5’ ends of their respective transcription units were compared by using run-on transcription assays. Nuclei were isolated from HeLa cells infected with Ad5 for increasing periods, and transcripts were labeled in run-on transcription reactions in the absence or presence of a concentration of α-amanitin sufficient to inhibit RNA polymerase II completely. The labeled RNA recovered from equal numbers of nuclei was fragmented and hybridized to immobilized viral DNAs containing major late (ML) and VA RNA 1 sequences transcribed by RNA polymerases II and III, respectively, as well as to a short, synthetic E2E DNA complementary to a sequence transcribed by both enzymes (positions −15 to +45). Typical results of these experiments are shown in Fig. 4A and B, and the data collected from several independent infections are summarized in Fig. 4C.

The rate of ML transcription by RNA polymerase II increased some 15-fold between 4 and 12 h after infection (Fig. 4A), in agreement with previous reports (30, 33, 46). Transcription of the VA RNA I gene by RNA polymerase III was also observed to be markedly more efficient during the late (12 h p.i.) than the early (4 h p.i.) phase of infection (Fig. 4B), again as expected (44). In contrast, much less marked changes in the rates of E2E transcription by either RNA polymerase II or RNA polymerase III with progression into the late phase of infection were detected (Fig. 4A and B). However, RNA polymerase II transcription of E2E sequences increased to a greater degree between 4 and 8 h after infection than did that by RNA polymerase III. To compare the efficiencies of E2E transcription by the two RNA polymerases, the relative rate of RNA polymerase II to that of RNA polymerase III transcription at various times was calculated from the results collected in several independent experiments. These data (Fig. 4C) indicated that E2E DNA sequences present in both transcription units are transcribed at essentially equal rates by RNA polymerases II and III from 8 h after infection. Furthermore, RNA polymerase III transcription was observed to be more efficient than that by RNA polymerase II at 4 h p.i. The large difference in the steady-state concentrations to which E2E mRNA and RNAs are made by RNA polymerase III therefore cannot be explained by preferential transcription of the finite number of E2E templates present in infected cell nuclei by RNA polymerase II.

Stabilities of E2E RNA species in Ad5-infected cells. The stabilities of the E2E RNAs synthesized by RNA polymerases II and III were next compared in order to investigate whether differences in this property could account for the lower concentrations of the products of RNA polymerase III transcription. In these experiments, transcription in Ad5-infected cells was inhibited by addition of actinomycin D and the concentrations of the E2E RNA species determined after increasing periods of incubation in the presence of the drug. Actinomycin D inhibits transcription upon intercalation into GC-rich sequences in the DNA template. Consequently, the size of the transcription unit to be targeted is an important determinant of the efficiency of actinomycin D inhibition. Because the E2E RNA polymerase III transcription unit is very small (Fig. 1), we first examined the inhibition of viral ML (RNA polymerase II), VA RNA1 (RNA polymerase III), and E2E (both enzymes) transcription by increasing concentrations of actinomy-
cin D. Addition of the drug to 10 μg/ml was found to inhibit completely transcription of these viral sequences by both RNA polymerases (data not shown). The cytoplasmic concentrations of E2E RNAs were therefore examined in Ad5-infected cells incubated for increasing periods in medium containing this concentration of actinomycin D by using RNase T1 protection. No decrease in the concentration of E2E mRNA (exon 1) was observed within a 2-h period following inhibition of transcription (Fig. 5). In contrast, the concentrations of both small E2E RNAs decreased rapidly during the same period. The maximal half-lifes of these E2E RNAs were estimated to be some 60 to 80 min (Fig. 5), assuming that transcription was inhibited as soon as actinomycin D was added to the culture medium. This value is almost certainly an overestimate, for the drug must enter cell nuclei and intercalate into viral DNA before transcription is inhibited. Regardless, these data indicate that E2E RNA polymerase III transcripts are considerably less stable than E2E mRNA.

FIG. 4. Relative rates of E2E transcription by RNA polymerase II and III during the infectious cycle. Run-on transcription reactions in nuclei isolated from Ad5-infected cells after the periods of infection indicated and hybridization and its quantification were as described in Materials and Methods. For each condition, equal numbers of nuclei were incubated in reactions lacking (RNA polymerases II and III) or containing (RNA polymerase III only) 2 μg of α-amanitin/ml, so that the rates of transcription by the two enzymes could be determined. Raw data from one experiment are shown in panels A (RNA polymerase II) and B (RNA polymerase III), while the mean values of the ratios of the rates of RNA polymerase II to RNA polymerase III determined in two independent infections are shown for E2E transcription in panel C. Because the VA RNA 1 gene lies within the ML transcription unit (47), it is transcribed by RNA polymerase II (A) as well as RNA polymerase III (B).

Competition for transcription of E2E templates in Ad5-infected cells. The closely similar rates at which E2E DNA sequences common to the two transcription units are transcribed by the RNA polymerases II and III (Fig. 4C) and the superimposition of promoter sequences (see the introduction) suggested that transcription by RNA polymerase II might limit transcription by RNA polymerase III, and vice versa. Infected cells contain a limited number of viral genomes, particularly before viral DNA synthesis begins, and formation of an RNA polymerase II initiation complex on its E2E promoter would
to be examined using a probe complementary to the transcription by both RNA polymerase III and RNA polymerase II mally terminate at the t2 site should read through into the RNA polymerase III transcriptional complexes that would nor-
RNA polymerase III E2E promoters but lack the t2 termination site for all sequences of both the RNA polymerase II and RNA polymerase III (Fig. 1). This organization suggested that some RNA polymerase III complexes terminate transcription at the t1 site, upstream of CAT DNA (Fig. 6A).

Previous studies have shown that substitution of each of the upstream RNA polymerase II promoter elements shown in Fig. 1 inhibits both basal and E1A protein-induced transcription of the E2E-CAT gene by RNA polymerase II in infected cells (32, 53). We therefore determined whether such mutations altered the relative efficiencies of transcription from their respective E2E promoters by RNA polymases II and III, using run-on transcription in isolated nuclei as described above. The results of such experiments, with CAT RNA syn-
thesized in cells infected by viruses carrying mutated E2E-CAT genes expressed relative to the value determined for cells infected by the wild-type E2E-CAT recombinant virus, are shown in Fig. 6B.

In agreement with previous observations (32, 53), substitu-
tion of the −25 to −34 sequence (which includes the 5’ end of the TATA sequence) or of either of the E2F binding sites (−35 to −46 and −55 to −66) impaired transcription of the CAT reporter gene by RNA polymerase II (Fig. 6A). However, in these direct assays of transcription we observed more-modest effects of the mutations than when either the production of active CAT enzyme or the steady-state concentrations of chi-
meric E2E-CAT RNAs were examined (32, 53). Furthermore, the −19 to −29 substitution, also in the vicinity of the TATA sequence, induced a modest increase in E2E CAT transcription by RNA polymerase II (Fig. 6A), rather than the large decrease in E2E-CAT mRNA synthesis described by Swan-
nathan and Thimmappaya (53). One possible explanation for these differences could be that, in the absence of any one of the proteins that bind to upstream sequences of the E2E promoter (Fig. 1), RNA polymerase II transcription is poorly processive beyond some point downstream of the 5’ end of the CAT probe used in experiments.

The results summarized in Fig. 6B indicated that one muta-
tion in the vicinity of the TATA sequence (−19 to −29 sub-
stitution) inhibited CAT transcription by RNA polymerase III. This effect was specific, for mutation of either of the E2F binding sites (−35 to −46 and −55 to −66 E2E-CAT viruses) led to reduced reporter gene transcription by RNA polymerase II but not by RNA polymerase III. Similar effects of TATA sequence and E2F-binding sites mutations on E2E transcription by RNA polymerase III in vitro reactions were observed previously (43).

Comparison of the relative rates of transcription from the wild-type and mutated E2E promoter by RNA polymerase II and RNA polymerase III (Fig. 6C) indicated that the rates of transcription by the two enzymes are reciprocally related. For example, the E2F-binding site mutations that impaired transcrip-
tion by RNA polymerase II induced an increase in the rate of CAT transcription by RNA polymerase III and conse-
sequently reduced significantly the ratio of RNA polymerase II to RNA polymerase III transcription (Fig. 6C). Conversely, the −19 to −29 substitution that inhibited RNA polymerase III transcription to a significant degree markedly increased this parameter (Fig. 6C).

DISCUSSION
The results reported here demonstrate properties of aden-


FIG. 5. Comparison of the stabilities of E2E RNAs synthesized by RNA polymerases II and III. Cytoplasmic RNA was prepared from HeLa cells infected with Ad5 for 12 h and then incubated in the presence of 10 μg of actinomycin D/ml for periods increasing from 0 to 120 min. Products of E2E transcription by RNA polymerases II and III were detected and quantified by using the RNase T1 protection assay described in Materials and Methods. The values determined for each RNA after increasing periods of actinomycin D chase are expressed relative to that measured in the absence of the drugs. The values shown represent the means of three independent experiments. The small increase in the concentration of E2E mRNA during the actinomycin D chase suggests that inhibition of transcription by the drug may not have been complete during the initial period. Consequently, the E2E RNAs made by RNA polymerase III may turn over even more rapidly than estimated (see text).

preclude binding of RNA polymerase III initiation proteins (and vice versa). To investigate whether such competition for E2E transcriptional templates indeed occurs in infected cells, we examined the effects of promoter sequence mutations on transcription by RNA polymerase II and III by using a series of recombinant viruses carrying ectopic E2E-CAT genes (32).

In the chimeric transcription units present in the nonessential E3 region of these viruses (Fig. 6A), E2E sequences from positions −284 to +62 are linked, via a short segment of plasmid DNA, to the CAT gene (32, 40). They therefore retain all sequences of both the RNA polymerase II and RNA polymerase III E2E promoters but lack the t2 termination site for RNA polymerase III (Fig. 1). This organization suggested that RNA polymerase III transcriptional complexes that would nor-
ternate at the t2 site should read through into the CAT coding sequence (Fig. 6A), allowing reporter gene transcrip-
tion by both RNA polymerase III and RNA polymerase II to be examined using a probe complementary to the 5’ end of the CAT coding sequence (Fig. 6A). The results of preliminary run-on transcription assays using nuclei isolated from cells infected for 12 h by the wild-type E2E-CAT virus indicated that this was indeed the case: CAT RNA synthesized by RNA polymerase III was detected readily. The ratio of RNA polymerase II to RNA polymerase III transcription of CAT se-
quences was some 50% higher than that of E2E sequences transcribed by both enzymes (Fig. 4C), as expected, because...
among viral genes transcribed by this enzyme: the products of such transcription are very unstable, and RNA polymerase III transcription can limit transcription from the superimposed E2E RNA polymerase II promoter.

Our initial studies of transcription of adenoviral E2E sequences by RNA polymerase III established that each of the two small RNA species made by this enzyme attains a maximal cytoplasmic concentration of only some 10 copies/cell (28). Direct comparison of the accumulation of E2E RNAs made by RNA polymerases II and III (Fig. 3) has now shown that the small E2 RNAs are present in significantly lower quantities than E2E mRNA throughout the initial period of the infectious cycle. This difference is not the result of E2E transcription by RNA polymerase III being less efficient than by RNA polymerase II, for the former enzyme transcribes E2E sequences common to the two transcription units at a rate equal to or greater than that of transcription by the latter (Fig. 4). Rather, the small E2 RNAs synthesized by RNA polymerase III are much less stable than E2E mRNA (Fig. 5). The short half-lives exhibited by E2E RNA polymerase III transcripts in actinomycin D chase experiments are consistent both with the detection of less-than-full-length species in RNase T1 protec-
tion (Fig. 2) and blotting (28) assays and with the greater relative concentration of such shorter E2E RNAs in infected cell RNA populations than among the products of in vitro transcription by RNA polymerase III (Fig. 2).

The E2E RNA polymerase III transcripts, molecules of ~45 and 90 nucleotides, represent the first examples of unstable viral RNAs made by this cellular enzyme. The first such RNAs to be identified, subgroup C adenoviral VA RNA 1 and VA RNA II have been estimated to accumulate to $10^6$ and $10^7$ copies/cell, respectively (37). The high concentration of VA RNA I is presumed to allow effective competition with other RNAs containing double-stranded regions for binding to double-stranded RNA-activated protein kinase (PK-R) and hence to allow blocking of activation of this important component of cellular antiviral defense mechanisms (37). The two small Epstein-Barr virus RNAs made by RNA polymerase III in latently infected cells are also present at high concentrations (some $10^5$ copies/cell) (1, 26), as are viral, tRNA-like RNAs made in cells infected by murine gammaherpesvirus 68 (7).

The high rate of production but rapid turnover of the small, adenoviral E2E RNAs could be a direct consequence of consumption (cleavage) of the primary transcripts as they fulfilled a catalytic or regulatory function. The contributions of RNA-mediated catalysis to essential cellular processes such as the processing of precursor RNAs (14, 22, 56) and to the replication of infectious agents such as viroids and hepatitis delta satellite virus (2, 14, 54) are well established. Furthermore, noncoding RNAs play a wide variety of regulatory roles, modulating reactions as diverse as transcription and translation (49). Indeed, inhibition of gene expression by small, interfering RNAs that target complementary mRNAs for rapid degradation appears to be a regulatory process that operates in essentially all eukaryotes (5, 10, 25, 59). There is therefore ample precedent for the hypothesis that the adenoviral E2E RNAs synthesized by RNA polymerase III participate directly in such a process, as yet undefined, that is important for the successful completion of the infectious cycle. Nevertheless, various observations suggest consideration of an alternative hypothesis, namely that it is E2E transcription by RNA polymerase III per se that facilitates viral replication.

Previous in vitro studies of E2E transcription by RNA polymerase II and III indicated that components of these transcription systems are in competition for access to specific internal promoter sequences (16). A similar phenomenon in infected cells has now been observed: mutations in upstream promoter sequences that inhibit transcription by RNA polymerase II stimulate transcription by RNA polymerase III, and vice versa (Fig. 6). It is highly unlikely that each of the substitutions that both inhibit transcription by one enzyme and stimulate transcription by the second happens to eliminate a negative regulatory sequence inhibiting the second enzyme. We therefore conclude that recognition of the E2E promoter by components of the RNA polymerase II initiation machinery and assembly of the preinitiation complex containing this enzyme block binding by RNA polymerase III initiation proteins, and vice versa. It therefore appears that transcription from one of the two E2E promoters limits transcription by the other RNA polymerase, even in cells infected at a fairly high multiplicity (20 PFU/cell) and thus containing multiple copies of the viral genome (19). Such effects would be expected to be even greater when cells contain fewer copies of viral DNA templates, as seems likely to be the case during natural infections.

The possibility that RNA polymerase III transcription from promoters present in repeated DNA sequences present in many eukaryotic genomes might regulate transcription of neighboring (or superimposed) class II genes has been suggested with some regulatory (8, 9, 11, 15, 34, 39, 41, 50, 51, 55). For example, it has been demonstrated that deletion of RNA polymerase III Alu transcription units located upstream of specific class II genes impairs transcription of the latter by RNA polymerase II in transient expression systems (31, 41). Conversely, transcription of tRNA genes by RNA polymerase III has been reported to repress strongly RNA polymerase II transcription of neighboring retrotransposons in Saccharomyces cerevisiae (29). Similarly, the "transcriptional competition" for access to common sequences of the E2E RNA polymerase II and III promoters described here suggests that the primary function of RNA polymerase III transcription might be to repress or damp transcription by RNA polymerase II. As noted previously, it is the rate of production of E2E mRNAs and the replication proteins which they encode that determines the length of the early phase of adenovirus infection. Inhibition or damping by RNA polymerase III transcription might therefore represent a mechanism to prevent premature entry into the late phase of infection, for example, until early proteins that function during the late phase, such as the E1B 55-kDa and E4 Orf6 proteins (13, 47), have accumulated to optimal concentrations. The immediate early E1A proteins that are required for efficient E2E transcription by RNA polymerase II in infected cells (32, 53) are also believed to optimize the host cell environment for viral DNA synthesis, by inducing entry into the S phase of the cell cycle (47). A mechanism that initially limits E2E transcription by RNA polymerase II could therefore also facilitate integration of the onset of viral DNA synthesis with the induction of cellular components that participate in this process.

The hypothesis that E2E transcription by RNA polymerase III serves to regulate the rate or timing of RNA polymerase II transcription implies that E2E RNAs I and II are merely by-products, serving no discrete molecular function. Their rapid turnover is consistent with this inference. The majority of transcriptional control signals of the Ad5 E2E RNA polymerase III transcription unit are conserved among other members of the Mastadenovirinae (Fig. 7). Indeed, an RNA polymerase III termination site, a run of $\geq$4 T residues flanked by G-C pairs (6; see also reference 23) at the t2 position, is significantly more strongly conserved than the E2E TATA-like sequence (Fig. 7). However, the t1 termination site that produces E2E RNA I is not present in all genomes (Fig. 7). Furthermore, Ad5 E2E RNA I is completely dispensable for replication of Ad5 in either transformed or normal human cells (R. Finnen and S. J. Flint, unpublished observations). These properties are consistent with the possibility that while E2E transcription by RNA polymerase III is important for adenovirus reproduction, the precise nature of the RNA(s) produced by this enzyme is not. This hypothesis predicts that E2E mutations that change substantially the products of RNA polymerase III transcription but do not alter the rates of E2E transcription by either enzyme will have no effect on viral replication. Conversely, if RNA polymerase III E2E transcripts perform a specific func-
such mutations should impair some specific step in the infectious cycle. Although straightforward in principle, tests of these predictions of the alternative hypotheses for the functional significance of the unusual adenoviral E2 transcription by RNA polymerase III are complicated by the fact that the segment of the r-strand of the viral genome complementary to the E2 transcriptional control region and RNA polymerase III transcription unit specifies an essential function. This r-strand sequence encodes the C terminus of the L4 33-kDa nonstructural protein, and this C-terminal portion of the L4 protein is required for assembly of any virus particles (17). Experiments to develop a genetic background suitable for mutational analysis of the function(s) of E2 transcription by RNA polymerase III during adenovirus replication are in progress.

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REFERENCES


FIG. 7. Comparison of Mastadenovirinae E2E sequences. Sequences related to the Ad5 E2E sequence from positions ~30 to +105 were first identified by a FASTA search of the GenBank viral DNA database. They were then aligned by using the Pile-up algorithm of the Wisconsin Package of SeqWeb. The most highly conserved sequences, a GA-rich sequence spanning the sites of initiation and the t2 termination site for RNA polymerase III transcription, are type set in white and highlighted in dark gray. The sequences corresponding to the positions of the A and B boxes of the Ad5 RNA polymerase III promoter are boxed, and TATA-like sequences are underlined. When present, RNA polymerase termination sites at the position of the Ad5 t1 site are highlighted in light gray.

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