Termination of Transcription by RNA Polymerase III from Wheat Germ*

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A novel system has been established to study transcription by RNA polymerase III. Purified wheat germ RNA polymerase III will initiate transcription on SV40 DNA templates amplified by polymerase chain reaction. Transcription initiates primarily at a thymidine 3 residues from a Poly T terminus at nucleotide 272 and terminates at a downstream T₆₅-T₆ sequence at nucleotides 21–28. Termination is enhanced if the RNA polymerase is caused to pause downstream of the A₆₅-T₆ sequence, but termination is unaffected by upstream sequences. Transcription of heteroduplex DNA templates with mismatches in the A₆₅-T₆ sequence indicates that termination is dependent upon the integrity of the A₆ sequence in the template strand and is only slightly affected by changes in the T₆ sequence of the non-template strand. However, templates containing 2'-deoxyuridine or 5'-bromo-2'-deoxyxuridine in place of thymidine in the non-template strand reduce termination efficiency, as does incorporation of 5'-bromouracil into RNA. There is no obvious correlation between DNA bending and termination efficiency.

Termination of transcription is an important step in gene expression. Minimally, termination consists of the release of the nascent RNA transcript and the dissociation of RNA polymerase from the DNA template. For this to occur efficiently, the activation energy for termination must approximate that for elongation (von Hippel and Yager, 1992). Transcription termination frequently involves sequences in the template and/or the transcript as well as accessory proteins that bind these sequences or the RNA polymerase. These elements must affect the relative activation energies for termination and elongation.

"Intrinsic" termination by Escherichia coli RNA polymerase (that is, termination independent of accessory proteins) requires secondary structure as well as a polyuridine sequence in the RNA transcript (Platt, 1986; Richardson, 1993). Formation of a stem-loop structure 7–10 nucleotides upstream of the 3' end of the nascent RNA signals the RNA polymerase to pause, and weak interactions between the DNA template and the RNA transcript facilitate the release of RNA from the transcription complex. Recent evidence suggests that for some RNAs, stem-loop structures may play only a minor role in termination, and the existence of an interaction between the RNA polymerase and the transcript that is sequence- or structure-specific has been conjectured (Telesnitsky and Chamberlin, 1989; Rice et al., 1991). Alternatively, other transcription termination events in E. coli utilize ρ factor. This protein binds to nascent RNAs at regions of high cytidine content and dissociates the RNA from the template at the expense of ATP hydrolysis (Richardson, 1993). Some proteins (exemplified by the λ phage N and Q proteins) specifically modify the transcription complex so that the RNA polymerase will not terminate (Richardson, 1993; Kerppola and Kane, 1991).

Termination of transcription by eukaryotic RNA polymerase I requires a protein that binds to a sequence element located between the ribosomal RNA gene repeats (Grunm et al., 1986; Bartsch et al., 1988; McStay and Reeder, 1990). The signal for transcription termination by RNA polymerase II includes a stretch of adenosines in the template strand (Reines et al., 1987; Dedrick et al., 1987; Kerppola and Kane, 1990, 1991; Enriquez-Harris et al., 1991). Flanking sequences around the termination site have some influence on termination efficiency (Dedrick et al., 1987; Kerppola and Kane, 1990). DNA bending also has been suggested to be part of a potential signal for termination (Gottlieb and Steitz, 1989b; Kerppola and Kane, 1990). Accessory proteins to RNA polymerase II are involved in termination, particularly those affecting the efficiency of elongation such as transcription factors IIF and IIS and the human immunodeficiency virus TAT factor (Bengal et al., 1991; Kao et al., 1987; Kato et al., 1992).

A termination signal for eukaryotic RNA polymerase III was first recognized as a short consecutive run of at least 4 or more adenosine residues on the template strand (Brown and Brown, 1976; Bogenhagen and Brown, 1981). Flanking sequences around this sequence may affect termination efficiency (Bogenhagen and Brown, 1981). One exception to this sequence was found in a salmon highly repetitive DNA, where a nonanucleotide sequence with alternating A and T residues causes termination (Matsumoto et al., 1989). Mutation of the second largest subunit of yeast RNA polymerase III results in reduced efficiency of transcription termination (James and Hall, 1990). Transcription termination is apparently catalyzed by RNA polymerase III alone, in the absence of other protein factors (Cozzarelli et al., 1983). The RNA-binding protein La has been suggested to stimulate termination of transcription by RNA polymerase III in HeLa extracts (Gottlieb and Steitz, 1989a); however, this has not been reproduced in other systems (Campbell and Setzer, 1992).

Virtually nothing is known about transcription termination by plant RNA polymerases. One obstacle for studying transcription by these enzymes is the lack of purified and defined functional transcription machinery that will faithfully initiate transcription. With highly purified animal RNA polymerases, one template that has been used to study transcription is a double-stranded DNA with a single-stranded 3'-poly(dC) tail (Kadesch and Chamberlin, 1982; Dedrick and Chamberlin, 1986; Kerppola and Kane, 1990; Campbell and Setzer, 1992). Here, we describe another type of DNA template that is easily

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generated by PCR. With such templates, we have studied transcription termination using purified wheat germ RNA polymerase III.

MATERIALS AND METHODS

DNA Templates for Transcription—PCR was used to generate transcription templates from different SV40 DNAs. One PCR primer (5'-CTGTGGAAATGCTGCAAGTCG) corresponds to the sequence of SV40 DNA from nucleotides 272 to 249; a second primer (5'-CCAAAAAGCCTCCTCCTCCTACTT) corresponds to the sequence from nucleotides 5195 to 5217 (Fig. 1). These PCR products share two features: 1) they have one terminus with the sequence of an SV40 fragment generated by NruI digestion that supports transcription initiation by purified RNA polymerase III (Watson et al., 1992), and 2) the wild-type SV40 DNA fragment contains an &T8 sequence that causes transcription termination from nucleotides 5195 to 5217 (Fig. 1). These PCR products were 12 base pair long and were obtained from Dr. P. Chambon (Pauly et al., 1992). DNAs substituted with dTTP instead of dTTP.

Heteroduplex DNAs with mismatches in the &T8 region were prepared by cloning the 294-bp KpnI-HindIII fragment of pSVlE, pSEG A5 or pSEG A2 into M13mp18 and M13mp19 vectors. Single-stranded M13 DNAs with SV40 DNA inserts were isolated and annealed in various combinations using complementary strands from pSV1E and pSEG A5 or pSEG A20. The DNAs were digested with HindIII and NruI, and the HindIII terminus was filled in with Klomow fragment of DNA polymerase I. The 272-bp HindIII/NruI fragment from each heteroduplex DNA was purified from a 2.5% agarose gel.

RNA Polymerase III—Wheat germ RNA polymerase III was purified from commercial wheat germ (Con-Agra Co., Kansas City, MO) according to the procedure of Jendrisak (1981) with the following modifications. After DEAE-Septarose CL-6B chromatography, phosphocellulose (Whatman P-11) was employed in place of the DEAE-cellulose column. Wheat germ RNA polymerase III purified in this manner was essentially free of RNA polymerase I and II activities. Nearly homogeneous RNA polymerase III was obtained by additional chromatography on a heparin-agarose column. Acanthamoeba RNA polymerase III was a gift from Dr. M. Zwick.

Transcription—Transcription reactions were carried out in 20 µl at 28 °C for 5 min unless otherwise stated. Standard reaction conditions were 12 mM HEPES (pH 8.0), 80 mM potassium acetate, 1.2 mM dithiothreitol, 5 mM magnesium acetate, 50 mM 2-mercaptoethanol, 1 µg/ml of heparin, 4 units (1.6 units/µl) of purified wheat germ RNA polymerase III (unit of enzyme is defined as the amount of enzyme required to incorporate 1 pmol of UMP into RNA at 28 °C within 10 min), and 0.5 pmol of template DNA. Typically, transcription reactions were initiated by the addition of ribonucleoside triphosphates at a final concentration of 500 µM for each of the three unlabeled ribonucleoside triphosphates and 25 µM for the α-32P-ribonucleotide triphosphate (10 Ci/mmol), unless otherwise stated. After incubation, reactions were stopped by the addition of 86 µl of stop solution (5.8 mM EDTA and 10 µg of E. coli tRNA in 50 mM sodium acetate (pH 5.5)) and extracted with phenol/chloroform.

Following precipitation with ethanol, RNAs were resuspended in 2.5 µl of H2O, denatured with 5 µl of 100% formamide at 90 °C for 5 min, and separated on a 10% denaturing polyacrylamide gel. Bands were visualized by exposure of the gel to Kodak X-ray film. Quantitation of band intensities on gels was performed on a Molecular Dynamics PhosphorImager.

Primer Extension—Primer extension analysis was performed following the protocol provided by Stratagene (La Jolla, CA). Transcription reactions were incubated for 20 min at 28 °C before the addition of 1 unit of RQ DNase I (Promega Biotec). Incubation was then shifted to 37 °C for 10 min and continued for another 5 min following the addition of 2 µl of proteinase K (2 mg/ml) along with 100 µl of stop solution (20 mM EDTA (pH 8.0), 1% sodium dodecyl sulfate, 0.2 M NaCl, and 0.25 mg/ml yeast RNA). Nucleic acids were extracted with phenol/chloroform, precipitated with ethanol, and then redissolved into 10 µl of annealing buffer (2 mM Tris-HCl (pH 7.8), 0.2 mM EDTA, and 250 mM KCl). Denaturation of the RNA and annealing with 32P-labeled primers was performed at 85 °C for 1.5 min and at 55 °C for 30 min, respectively. 40 µl of extension solution (62.5 mM Tris-HCl (pH 8.3), 1.25 mM MnCl2, 125 µg/ml actinomycin D, 12.5 mM dithiothreitol, and 0.33 mM each dATP, dGTP, dCTP, and dTTP) and 20 units of reverse transcriptase (Stratagene) were mixed with the RNA sample and incubated at 42 °C for 1 h. Single-stranded cDNA products were ethanol-precipitated and subjected to electrophoresis on a 10% denaturing polyacrylamide gel.

Bio-Gel P-60 Column Chromatography—A transcription reaction was carried out in a 100-µl reaction volume using the conditions described above. After 5 min of incubation, 1.5 µl of 0.5 mM EDTA was added, and the reaction mixture was directly loaded onto a Bio-Rad Bio-Gel P-60 column (0.6 x 10 cm) equilibrated with transcription buffer (12 mM HEPES (pH 8.0), 80 mM potassium acetate, 1.2 mM dithiothreitol, 5 mM...
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Fig. 2. Transcription analysis of pSVB-3 template by RNA polymerase III preparations. P-11 wheat germ RNA polymerase III was purified through a phosphocellulose P-11 column, and heparin RNA polymerase III was purified through both P-11 and heparin-agarose columns. Acanthamoeba castellanii RNA polymerase III was a homogeneous preparation. The concentration of the limiting nucleotide (nt.) in each reaction was 25 μM, and that of the other three nucleotides was 500 μM each. Terminated and readthrough products were determined as described above.

Analysis of DNA Bending—Template DNAs of the same length (pSV1E, pSV1E-dU, pSV1E-dB, pSEG A5, pSEG A7, pSEG A20, pSEG A21, pSEG A22, pSEG A23, and pSEG A24) were subject to electrophoresis on a 12% nondenaturing polyacrylamide gel at 4°C. The mobilities of pSV1E (equivalent to pSEG A0), pSEG A5, pSEG A20, pSEG A21, pSEG A22, and pSEG A23 (Paul et al., 1992) were used in the calculation of the migration anomalies of pSV1E-dU, pSV1E-dB, pSEG A7, and pSEG A24 DNAs.

RESULTS

Watson et al. (1984) observed that with PvuII-digested SV40 DNA and purified calf thymus RNA polymerase III, in vitro transcription was initiated at the end of a specific SV40 fragment and terminated at an A<sub>T</sub><sub>Φ</sub> sequence. We extended this observation to highly purified wheat germ RNA polymerase III and furthermore have found that PCR products of the SV40 fragment also serve as templates. A large variety of SV40 mutants with nucleotide substitutions in the region that promotes termination have been generated and studied by us and others, and using PCR amplification of these DNAs greatly simplifies preparation of templates for transcription.

When PCR products of the SV40 DNA template pSVB-3 were transcribed with purified wheat germ RNA polymerase III, two major products were produced of ~242 and 320 nucleotides. Experiments described below indicate that the 242-nucleotide RNA transcript is terminated near the A<sub>T</sub><sub>Φ</sub> sequence and that the 320-nucleotide transcript is a readthrough RNA. The same two products were produced by several preparations of RNA polymerase III of different purity or source (Fig. 2). Limiting nucleotides altered the efficiency of transcription and the relative amounts of these products. The reason for this effect is analyzed below.

To identify the template strand from which these RNAs were transcribed, primer extension analysis of the transcripts was performed. Primer 1 (Fig. 3A) was designed to hybridize to RNAs initiated from the non-PvuII end of the template; RNAs initiated from the PvuII end should hybridize to primer 2. RNAs were transcribed by RNA polymerase III using, in the reaction mixture, all four nucleotides at 500 μM or a single limiting nucleotide at 25 μM and the other three at 500 μM. The transcripts were annealed to the 32P-labeled primers, and the primers were extended by reverse transcriptase. After denaturing polyacrylamide gel electrophoresis, only products resulting from synthesis with primer 2 were observed, indicating that transcription was initiated only at the PvuII end of the template (Fig. 3B). To determine the precise site at which transcription begins on these templates, we annealed primer 2 to the transcripts, extended the primers with reverse transcriptase, and then fractionated the cDNAs on a denaturing high resolution polyacrylamide gel with DNA markers corresponding to the sequence of the template (Fig. 4). This analysis indicated that a uridine residue was the first nucleotide incorporated into RNA when all four nucleoside triphosphates were at high concentrations (500 μM) or with limiting ATP or CTP (25 μM) in the reaction mixture. With limiting ATP, a minor portion of RNAs initiated at the G residue complementary to C<sup>270</sup>. With limiting UTP, transcription was very inefficient, and the initiation site shifted downstream to the G residue complementary to C<sup>267</sup>.

To determine the importance of different sequence elements for transcription termination, PCR products of several mutant DNAs with alterations in this region were generated and tran-
Fig. 3. Determination of transcription direction. A, schematic representation of the strategy employed. The A8:T8 sequence in the pSVB-3 DNA template is represented by a filled box, and 21- and 72-base pair repeats are represented by open boxes. Long horizontal arrows indicate the RNA transcripts. Primers used for primer extension analysis are shown by short horizontal arrows. (Primer 1 will only hybridize to the RNA transcribed from the non-PvuII terminus, whereas primer 2 is specific for the RNA transcribed from the PvuII end.) Putative cDNA products are drawn as short horizontal lines with predicted lengths labeled underneath. B, products of primer extension assay. Shown is an autoradiograph of an 8 M urea, 10% polyacrylamide gel used to fractionate cDNA products of transcription. Sizes of transcripts measured with DNA markers are indicated. n.t. (or nt), nucleotide.

scribed (Fig. 5). Deletion of the A8:T8 sequence and adjacent 21-bp repeat elements in pX113 DNA caused only readthrough products to be synthesized. However, a DNA with the A8:T8 sequence but without the 21-bp repeat elements (p21-0) supported termination. Also notably, a construct with a long string of thymidines in the template strand preceding the A8:T8 sequence (pAN8) still terminated at the latter (Fig. 5). These results indicate that the A8:T8 sequence is required for termination and that the polarity of an A:T sequence is important for termination. The RNA products produced in these reactions terminated near the A8:T8 sequence could result either from an actual (intrinsic) termination event or from physical disruption of a paused transcription complex, e.g. by the phenol/chloroform extraction that follows the transcription reaction. To distinguish between these possibilities, a transcription reaction was placed directly (without phenol extraction) onto a Bio-gel P-60 column, and the elution of the RNA molecules was monitored so as to determine whether the RNAs eluted with the transcription complex or free of templates and RNA polymerase. Since the exclusion limit of this column is ~60,000 Da, any ternary transcription complexes should be present in the excluded volume, whereas the free RNA transcripts (both terminated and readthrough products) should be found in the included volume. The observed elution of terminated and readthrough products in the included volume (Fig. 6) demonstrates that they are free of the template and RNA polymerase.

Why is the efficiency of transcription termination greater with limiting ATP than with limiting CTP? To study this, both CTP and ATP were made limiting in a transcription reaction to see how limiting one nucleotide alters the effect of limiting the other. Under these conditions, termination was as efficient as with limiting ATP alone (Fig. 7). To explore whether ATP is required as an energy source for the RNA polymerase III to overcome a termination event or to bypass a termination site, ATP analogs were used in the transcription reaction to supplement limiting ATP. AMP-CP can be used as an energy source (but not as a substrate for transcription), whereas AMP-PNP serves primarily as a substrate for transcription (Yount et al., 1971; Penningroth et al., 1980). Transcription of the pSVB-3 template was carried out in the presence of these analogs at a concentration of 475 μM, with or without ATP at 25 μM (when no
ATP was added to the reaction, $[\alpha^{-32}P]CTP$ (25 μM) was used to label transcripts) (Fig. 7). We observed that as long as ATP was limiting, termination efficiency was high, regardless of whether or not the ATP analogs were present as supplements (Fig. 7, lanes 1, 3, 4, and 5). In the reaction with only AMP-CPP (without ATP), no RNA products were made (lane 6), and comparison between the reactions with or without high AMP-PNP levels suggested that AMP-PNP is a poor substrate for RNA synthesis (Fig. 7, lanes 1, 3, 5, and 7). These results suggest that the primary effect of limiting ATP is on its role as a substrate for transcription and not on a subsidiary role relating to the termination reaction.

An explanation for the effect of limiting ATP upon termination might be provided by better definition of the terminator sequence. To examine further the role of the A₈:T₈ sequence and the sequence immediately upstream and downstream upon transcription termination, use was made of an extensive series of SV40 mutants containing single or double base changes in this sequence (Fig. 8) (Pauly et al., 1992). Transcription of pSEG A1 and pSEG A2 showed the same termination pattern as that of the wild-type DNA (compare with Fig. 2). However, as the nucleotide substitutions moved into the A₈:T₈ sequence (in pSEG A3 and pSEG A4), more and more readthrough products were made, until with template pSEG A5, all of the RNAs transcribed were readthrough products. Transcription termination was similar with template DNAs containing double (pSEG A2 and pSEG A3) and single (pSEG A14 and pSEG A16) base substitutions, indicating that the position of the base substitution is more important than the number of bases substituted. A plot correlating termination efficiency with mutation site summarizes these results (Fig. 9). All of the mutations that cause more than a 2-fold decrease in termination, i.e. pSEG A3, pSEG A4, pSEG A5, pSEG A16, pSEG A20, pSEG A21, r Seg A22, and pSEG A23, fall exactly within the boundary of the A₈:T₈ sequence. Other mutations, such as pSEG A1, pSEG A2, pSEG A7, pSEG A9, pSEG A10, pSEG A11, pSEG A14, pSEG
A24, and pSEG A26, do not have a similar influence on the termination efficiency and do not occur within the $A_8:T_8$ sequence. These data also indicate that the minimal sequence for efficient termination by wheat germ RNA polymerase consists of $A_5:T_8$.

Inspection of the dependence of termination upon limiting ATP concentration with the various mutants indicated that changing the deoxyadenosine following the $A_8:T_8$ sequence in the non-template strand to deoxycytidine (as in pSEG A24) altered the termination pattern observed with limiting ATP versus that observed with limiting CTP (Fig. 9). This suggests that pausing of the RNA polymerase following the eight consecutive A residues on the template strand might be responsible for the enhancement of termination by low ATP concentration. To investigate this possibility further, a study of transcription termination was carried out with wild-type DNA using limiting CTP and varying ATP concentrations from 0 to 500 $\mu$M. Since transcription products linearly increase within the first 12 min of transcription, we sampled the transcription reactions after 5 min of incubation. A plot of the percentage of readthrough products versus ATP concentration for this template yielded a curve with a slightly sigmoidal shape (Fig. 10, curve pSVB-3). However, transcription of pSEG A24, in which the adenosine residue following the $A_8:T_8$ sequence in the non-template strand is replaced by a cytidine, responded very differently to the concentration of ATP, as its termination efficiency was only slightly affected. Alternatively, when the CTP concentration was varied (using ATP as the labeled nucleotide), transcription of pSEG A24 demonstrated a strong dependence of termination efficiency upon CTP. To be sure that this effect was due solely to the single substitution in pSEG A24, a wild-type SV40 template exactly analogous to pSEG A24 except for the single substitution (pSV1E) was transcribed and found to give essentially the same result as pSVB-3. When these data were replotted in a double-reciprocal fashion, half-maximal readthrough was found to occur at 125 $\mu$M CTP, which is approximately the $K_m$ for CTP for wheat germ RNA polymerase III.$^3$ This result indicates that a determining factor for termination efficiency is the availability of the nucleotide following the $A_8:T_8$ sequence and underscores the importance of the $A_8:T_8$ sequence and downstream nucleotides for transcription termination.

As a means to examine the importance of each strand of the $A_8:T_8$ sequence for transcription termination, we constructed heteroduplexes in which one strand has a sequence change (Fig. 11A). Transcription of these heteroduplex DNA templates resulted in the synthesis of an RNA terminated at the $A_8:T_8$ sequence and three longer RNA products (Fig. 11B). The longer RNAs are readthrough products of the $A_8:T_8$ sequence, where one is the normal readthrough RNA and the other two are the products terminated at A runs close to the end of the templates (those two longer terminated transcripts were not seen with transcription using PCR-generated templates because the PCR-generated templates are shorter than the heteroduplex DNA templates and do not contain extra A runs).

Transcription of the four heteroduplex DNA templates with base mismatches in the $A_8:T_8$ region showed diverse termination patterns with termination efficiencies between those of pSV1E and pSEG A5, from 90.5 to 9.9% with limiting ATP and from 47.5 to 3.4% with limiting CTP. For example, template T/A2C has a normal $T_8$ sequence and an $A_8$ sequence with the fifth and sixth adenosines replaced by 2 cytidines. Termination efficiency of this template is depressed 4–10-fold with limiting ATP or CTP, respectively (Fig. 11, A and C). Transcription of

$^3$ X. Wang and W. R. Folk, unpublished data.
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**Fig. 6. Size fractionation of transcription products.** A marker RNA (−296 nucleotides (n.t.)) and the terminated (T; −242 nucleotides) and readthrough (RT, −320 nucleotides) RNAs transcribed by wheat germ RNA polymerase III were fractionated as described under “Materials and Methods.” Material excluded from the column eluted near 0.8 ml of the eluent. Free nucleotides were eluted in the peak at an elution volume of 3 ml.

**Fig. 7. Effect of ATP analogs on transcription termination.** Transcription was performed with pSVB-3 DNA as a template and with varying concentrations of ATP and CTP or with ATP analogs. GTP and UTP concentrations were 500 μM each. RNA products were electrophoretically separated on a 10% denaturing polyacrylamide gel. Lane 1, 25 μM ATP and 500 μM CTP; lane 2, 25 μM CTP and 500 μM ATP; lane 3, 25 μM each ATP and CTP; lane 4, 25 μM ATP, 500 μM CTP, and 475 μM AMP-CPP; lane 5, 25 μM ATP, 500 μM CTP, and 475 μM AMP-PNP; lane 6, 475 μM AMP-CPP, 25 μM CTP, and no ATP; lane 7, 475 μM AMP-PNP, 25 μM CTP, and no ATP.

The involvement of the sequence of the template strand in termination might be explained by the formation of weak interactions between the poly(rU) sequence of nascent RNA and the poly(dA) sequence of the template strand, which would help cause the RNA to dissociate from the DNA template. (There is evidence supporting a rather short sequence of the RNA:DNA hybrid in the yeast RNA polymerase III and the E. coli RNA polymerase transcription complexes (Kassavetis et al., 1992; Kainz and Roberts, 1992).) To explore this possibility, we employed 5'-BrUTP in a transcription reaction, as RNA with this analog forms a more stable hybrid with DNA (Dedrick et al., 1987; Chamberlin et al., 1963). A decrease of 12–30% in termination efficiency was observed when this analog was incorporated (Table I), in general support of the importance of the instability of the dA:rU hybrid for termination to occur during transcription.

To explore further the importance of the A₈:T₈ sequence and the poly(dA) sequence of the template strand, which would help nearby sequences for transcription termination, we examined whether its capacity to bend is correlated with termination.
AT-rich regions in double-stranded DNA frequently cause structural changes in DNA (Diekmann et al., 1992; Koo et al., 1986), and it has been suggested that DNA bending, either intrinsic or that induced by protein binding, may play a role in termination (Kerppola and Kane, 1990; Gottlieb and Steitz, 1989a). Pauly et al. (1992) have correlated DNA sequence with
also serve as templates for transcription. This builds upon the work of Watson et al. (1984), who observed that a SV40 DNA strand affects transcription termination.

As another means to examine the template requirements for termination, we examined DNAs in which base analogs were incorporated during the PCR amplification. DNA templates containing base analogs such as dUMP or BrdUMP have altered physical-chemical properties, but are unchanged in DNA bending (<1%) (Fig. 12). Remarkably, the termination efficiency observed with these templates was reduced by 40–60% (Table II). This might result because the structure of the DNA duplex is altered, or the non-template T₈ sequence may be directly involved in transcription elongation or termination. At present, we cannot distinguish between these alternatives. However, we note that dU:dA is less stable and dB:dA is more stable than dA:dT, and since both dU and dB incorporation reduces termination efficiency, the stability of the duplex is not likely to be the cause of the effect of these analogs upon termination. Additional studies will be required to determine how the non-template strand affects transcription termination.

**DISCUSSION**

It is difficult to study transcription with highly purified eukaryotic RNA polymerases because these enzymes do not initiate transcription correctly without accessory proteins. One approach to by-pass this difficulty (first developed by Kadesch and Chamberlin (1982)) employed a double-stranded DNA template with a single-stranded poly(dC) tail at its 3′-end. Transcription is initiated specifically at the junction of double- and single-stranded DNA (Dedrick and Chamberlin, 1985), and such templates have been used to study transcription by RNA polymerases from a variety of sources, including RNA polymerase III from Xenopus laevis (Campbell and Setzer, 1992).

We have found that PCR-generated fragments of SV40 DNA also serve as templates for transcription. This builds upon the work of Watson et al. (1984), who observed that a SV40 DNA fragment with a PacI end was specifically transcribed by purified calf thymus RNA polymerase III (but not by purified RNA polymerase II). In our work, we have used the same SV40 DNA sequence, amplified by PCR, as transcription templates to study transcription termination by highly purified wheat germ RNA polymerase III. Advantages of this approach are that there is no need to deal with the poly(dC) tailing of the DNA, and that with PCR amplification, it is relatively easy to obtain large quantities of DNA templates from a variety of SV40 mutants that are available from our laboratory and others.

That similar patterns of transcription are observed with preparations of RNA polymerase III of different degrees of purity or source suggests that accessory proteins contaminating the RNA polymerase preparations do not significantly affect initiation and termination on these templates. Addition of protein fractions from crude extracts of wheat germ did not change the transcription patterns, nor did addition of recombinant human La protein or antibodies directed against human La protein (data not shown).

With limiting UTP or GTP, transcription efficiency was greatly reduced, most likely because of the requirement for UTP and GTP for initiation. Of the first 29 nucleotides in the non-template strand, 24 are either thymidine or guanosine residues. Thus, transcription with limiting UTP or GTP probably aborts, as the shortage of either of these substrates may destabilize the initiation complex.

Low nucleotide concentrations are known to promote termination of transcription (Bogenhagen and Brown, 1981; Fisher et al., 1985; Chan and Landick, 1989; Wiest and Hawley, 1990). With these templates, we observed that when ATP was limiting, the majority of the RNAs were terminated at an A₈:T₈ sequence. With limiting CTP, about two-thirds of the transcripts resulted from termination at this sequence, and one-third of the transcripts were readthrough products.

Prokaryotic ρ-independent terminators contain an RNA stem-loop structure rich in GC content that seems to be important for transcription termination (Cheng et al., 1991; Rosenberg and Court, 1979). Since the wild-type SV40 DNA template has three 21-bp repeated sequences that are rich in GC content and have the potential to form stem-loop structures upstream of the termination site, we examined the transcription of a DNA template lacking these GC-rich sequences. With several such
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A

| DNA template | Termination Efficiency |
|--------------|------------------------|
| pSV1E        | 97.0%                  |
| pSEG A5      | 72.6%                  |
| pSEG A20     | 54.8%                  |
| T/A2C        | 54.8%                  |
| T/AC         | 75.9%                  |
| T2G/A        | 61.1%                  |
| TG/A         | 81.3%                  |

B

DNA templates, we did not observe any significant changes in the pattern of termination, suggesting that RNA secondary structure preceding the A8-T8 termination sequence is not important for transcription termination by wheat germ RNA polymerase III. Transcription of DNA templates with a tRNA<sup>Trp</sup> gene inserted in place of the 21-bp repeats also gave a similar result (data not shown).

Transcription of SV40 DNA templates with limiting nucleotide concentrations produced distinctive termination patterns, and we have shown that the sequence following the A8:T8 sequence plays a key role in determining the termination efficiency. When an adenosine is located immediately downstream of the A8:T8 sequence in the non-template strand, transcription

C

| Table I Effect of incorporation of 5'-bromouridine monophosphate on termination efficiency |
|-----------------------------------------------|-----------------|-----------------|-----------------|
| DNA template | Nucleotide incorporated | Limiting nucleotide | Termination efficiency |
|---------------|-------------------------|-------------------|------------------------|
| pSV1E         | UMP                     | ATP               | 97.0%                  |
| pSV1E         | UMP                     | CTP               | 72.6%                  |
| pSV1E         | BrUMP                   | ATP               | 56.5%                  |
| pSV1E         | BrUMP                   | CTP               | 54.8%                  |
| pAN3          | UMP                     | ATP               | 51.8%                  |
| pAN3          | UMP                     | CTP               | 51.8%                  |
| pAN3          | BrUMP                   | ATP               | 34.9%                  |
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FIG. 12. DNA bending and termination efficiency. The mobility anomalies for pSV1E, pSEG A5, pSEG A7, pSEG A20, pSEG A21, pSEG A22, and pSEG A23 were derived from Pauly et al. (1992), and those for pSEG A24, pSV1E-dU (template DNA with thymidines replaced by deoxyuridine), and pSV1E-dB (template DNA with thymidines replaced by 5'-bromodeoxyuridine) were determined by electrophoresis of the DNAs on a 15% native polyacrylamide gel at 4°C, and the mobilities were compared with those of pSV1E and pSEG A5. The percentage of termination for each of the DNA templates was determined as described under "Materials and Methods." Mobility anomalies of these template DNAs are plotted on the abscissa, and the percentage of terminated products over total of readthrough and terminated products for these DNAs is represented by the ordinate. *, transcription reactions carried out with limiting ATP; O, reactions performed with limiting CTP. Low A, limiting ATP (25 μM); Low C, limiting CTP (25 μM).

TABLE II

| DNA        | Nucleotide analog | Termination efficiency |
|------------|------------------|------------------------|
|            |                  | Limiting ATP | Limiting CTP |
| pSV1E      | dU               | 96.7         | 73.2         |
| pSV1E      | dU               | 63.5         | 15.2         |
| pSV1E      | dB               | 77.6         | 47.7         |
| pSEG A23   | dU               | 75.9         | 51.4         |
| pSEG A23   | dB               | 30.9         | 7.8          |
| pSEG A23   | dB               | 66.0         | 40.7         |

with limiting ATP (25 μM) resulted in >90% termination. When the dA following the As:T8 sequence in the non-template strand is replaced by a dC (as in pSEG A24), >90% of transcription terminates with limiting CTP. Because of the presence of a second dA downstream of the As:T8, a termination efficiency above 90% was also observed with this template while using limiting ATP.

An intrinsic limit of readthrough of the As:T8 sequence was estimated to be ~30%. Measurement of the dependence of termination upon ATP concentration with pSVB-3 and pSV1E DNAs revealed a slightly sigmoidal curve, indicating that a decrease in termination efficiency with increased ATP concentration occurs in a nonlinear fashion. Although the implication of the nonlinearity of this curve is not clear, this might reflect interactions between the RNA polymerase and DNA template at the termination site. Examination of the sequence following the As:T8 region reveals on the non-template strand two sites where RNA polymerase III might pause due to the depletion of ATP (Fig. 13). When ATP is limiting, these two sites might affect termination sequentially and could be the basis for the slightly sigmoidal-shaped curve that is observed. When the dA at the first site is replaced by a dC, a hyperbolic curve is observed with limiting CTP or with limiting ATP, further suggesting that the sigmoidal curve is due to these two sites.

Transcription of dU- or dB-substituted DNAs and of heteroduplex DNAs suggested that both strands of DNA in the As:T8 terminator play a role in transcription termination. The non-template DNAs with T8 sequences interrupted by one or two base substitutions did not show significant reduction in termination efficiencies, but did so when all the thymidines in the T8 sequence were replaced by either dU or dB. In contrast, muta-
tional substitution of 1 or 2 deoxyadenosines in the template strand A₉ sequence essentially eliminated transcription termination. These results suggest that although the T₉ sequence in the non-template strand can affect termination, the A₉ sequence in the template strand is much more important. One possible reason for this latter effect is that transcription termination may be promoted by the instability of dA+U in the transcription complex. This receives support from our observation that incorporation of 5'-bromouracil into RNA diminishes termination efficiency.

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