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Analysis of the Signals for Transcription Termination by Purified RNA Polymerase II†

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ABSTRACT: Eukaryotic RNA polymerase II recognizes certain DNA sequences as effective signals for transcription termination in vitro. Previously, we have shown that such termination occurs within T-rich sequences; however, not all T runs stop the enzyme nor is the efficiency of termination correlated with the length of the T run. Here we have investigated the sequence elements that signal transcription termination by purified RNA polymerase II. We have examined terminators located within introns of the human histone H3.3 gene and the human c-*myc* gene. Deletion analysis of the H3.3 termination region indicates that the sequences between −6 and +24 relative to the strongest termination site are sufficient to cause transcription termination. The minimal termination signal at this site has been localized to the sequence TTTTTTTC-CCTTTTTT in the nontranscribed strand. A similar but nonidentical sequence has been defined for the c-*myc* termination site. Since RNA polymerase II terminates transcription only within the first run of T residues in these sequences, at least part of the termination signal lies in downstream nontranscribed DNA sequences. Restriction fragment mobility analysis indicates that the H3.3 termination region contains a bend in the DNA helix. Oligonucleotides containing the minimal termination signals also cause restriction fragments to migrate with anomalous mobility. A region of the SV40 genome containing a previously characterized bend also causes RNA polymerase II to terminate transcription. We suggest that a structural element causing a bend in the DNA helix may be part of the signal for transcription termination by purified RNA polymerase II.

Transcription termination is an important mechanism for the regulation of gene expression in both prokaryotic and eukaryotic organisms. Termination in eukaryotes occurs downstream from the processing sites which generate the mature 3′ ends of transcripts for all three nuclear RNA polymerases (Platt, 1986; Sollner-Webb & Tower, 1986; Geiduschek & Tocchini-Valentini, 1988). In addition, intragenic blocks to transcript elongation have been detected in several genes transcribed by RNA polymease II (Bentley & Groudine, 1986; 1988; Eick & Bornkamm, 1986; Cesarman et al., 1987; Kerppola & Kane, 1988; Reines et al., 1987, 1989; Nepveu & Marcu, 1986; Wright & Bishop, 1989; Evans et al., 1979; Maderious & Chen-Kiang, 1984; Mok et al., 1984; Nevins & Wilson, 1981; Hay et al., 1982; Pfeifer et al., 1983; Skarnes et al., 1988; Kao et al., 1987; Resnekov & Aloni, 1989; Bender et al., 1987; Fort et al., 1987; Lattier et al., 1989; Bhat & Padmanaban, 1988). In some cases, these transcriptional blocks may regulate gene expression through conditional transcription termination (Bentley & Groudine, 1986; Eick & Bornkamm, 1986; Cesarman et al., 1987; Mok et al., 1984; Kao et al., 1987).

The study of transcription termination in eukaryotes is complicated by the rapid posttranscriptional processing of primary transcripts. Nuclear run-on analysis has been used to identify regions that block transcript elongation in several genes. However, with this method, it is difficult to identify the specific sites where RNA polymerase stops, and this method cannot distinguish between transcription termination and transcriptional pausing. It is also difficult to exclude the possibility that posttranscriptional processing destabilizes the nascent transcript. In order to circumvent these problems, we have used a defined in vitro transcription system that allows efficient transcription initiation by purified RNA polymerase II in the absence of accessory factors (Kadesch & Chamberlin, 1982). Using this transcription system, we have identified sites, which we designate intrinsic termination sites, where purified RNA polymerase II ceases elongation and releases the nascent

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transcript (Kerppola & Kane, 1988; Reines et al., 1987; Dedrick et al., 1987). Intrinsic termination sites are found in regions that block transcript elongation in vivo (Bentley & Groudine, 1988; Kerppola & Kane, 1988; D. Wells, personal communication). One such site is important in regulating c-*myc* gene expression (Bentley & Groudine, 1986, 1988; Eick & Bornkamm, 1986; Cesarman et al., 1987). Thus, intrinsic termination sites represent at least one class of transcriptional blocks used by the cell.

Intrinsic termination sites generally occur in T-rich sequences, frequently with a T run at the termination site (Kerppola & Kane, 1988; Reines et al., 1987; Dedrick et al., 1987). However, not all T runs function as intrinsic termination sites nor is the efficiency of transcription termination correlated with the length of the T run (Kerppola & Kane, 1988; Reines et al., 1987; Dedrick et al., 1987). Thus, additional sequences are required to cause purified RNA polymerase II to recognize a particular T run as a termination site. In order to identify these additional sequences, we analyzed in detail the sequences in the first intron of the human H3.3 gene and in the first intron of the human c-*myc* gene that cause RNA polymerase II to terminate transcription in these introns.

Materials and Methods

Enzymes. Calf thymus RNA polymerase II was purified to >90% homogeneity by a modification (Kerppola & Kane, 1988) of the method described by Dedrick and Chamberlin (1985). This enzyme preparation has a specific activity of approximately 100 units/mg and contains approximately 30% active molecules. *Escherichia coli* RNA polymerase was purified as described by Gonzales et al. (1977).

Terminal deoxynucleotidyl transferase was purchased from Ratliff Biochemicals, Los Alamos, NM. The Klenow fragment of DNA polymerase I and *Bal*31 nuclease were purchased from Bethesda Research Laboratories. Ribonuclease A was purchased from Sigma Chemical Co. Modified T7 DNA polymerase (Sequenase) was purchased from United States Biochemical Corp. Restriction endonucleases were purchased from Bethesda Research Laboratories, International Biotechnologies Incorporated, and New England Biolabs, and the digestions were performed under conditions recommended by the supplier.

Plasmid Constructions. Plasmid pTK200 was constructed by inserting the *Hin*dIII–*Sau*3A fragment from plasmid pSV2CAT (Gorman et al., 1982), which contains the coding region for chloramphenicol acetyltransferase, into the *Sal*I–*Xba*I gap of plasmid pRSV4 (provided by G. Chu and P. Berg, Stanford University), which contains the Rous sarcoma virus LTR promoter upstream of an SV40 hybrid mini-intron (Okayama & Berg, 1983) and the SV40 early region polyadenylation site. Plasmid pTK201 was derived from plasmid pTK200 by cleaving the *Bam*HI site downstream of the chloramphenicol acetyltransferase gene and filling in with the Klenow fragment of DNA polymerase I. Plasmid pTK202 was constructed by inserting the *Eco*RV–*Stu*I fragment of plasmid pIBI76 (provided by International Biotechnologies, Inc., New Haven, CT), which contains a polylinker sequence and phage SP6 and T7 promoters, into the *Bam*HI site in the mini-intron of plasmid pTK201 such that the phage SP6 promoter reads in the same direction as the RSV promoter. Plasmids pTK242A and pTK242AA were constructed by inserting the *Taq*I fragment from plasmid pHuH3-640 (Reines et al., 1987), which contains the intrinsic termination sites in the first intron of the H3.3 gene, into the *Acc*I site of plasmid pTK202 in the sense direction relative to the RSV promoter. Plasmid pTK242A contains a single copy of the termination region while plasmid pTK242AA contains a tandem duplication of this region.

Deletions were introduced into the H3.3 termination region in plasmid pTK242A by limited digestion with *Bal*31 nuclease after cleavage with either *Bst*EII (5′ deletions) or *Nsi*I (3′ deletions). The ends were were filled in with the Klenow fragment of DNA polymerase I, ligated with linkers containing a recognition site for *Bgl*II, digested with *Bgl*II, and recircularized with T4 DNA ligase. The sequences of the deletion derivatives were determined by the chain termination method using modified T7 DNA polymerase (Tabor & Richardson, 1987).

Double-stranded DNA oligomers for insertion into cloning vectors were generated by mutually primed synthesis (Hill et al., 1987). Single-stranded oligonucleotides were designed with a self-complementary 3′ palindrome and restriction endonuclease recognition sites flanking the sequence to be inserted. DNA oligonucleotides were synthesized by the solid-state phosphoramidite method using a Biosearch 8750 DNA synthesizer. Full-length oligonucleotides were separated from shorter products by polyacrylamide gel electrophoresis and recovered from gel slices by diffusion into 10 mM Tris-HCl (pH 8)/1 mM EDTA. The oligonucleotides were self-annealed by heating to 70 °C for 5 min and incubating at room temperature for 60 min. Deoxyribonucleotides were added to 500 μM, and the annealed oligonucleotides were elongated with 5 units of the Klenow fragment of DNA polymerase I for 180 min at 23 °C. The double-stranded oligomers were digested with the restriction endonucleases cleaving at the flanking recognition sites, and were ligated to plasmid pTK202 cleaved with the same restriction endonucleases. The sequences of the oligonucleotide inserts were determined by the chain termination method using modified T7 DNA polymerase (Tabor & Richardson, 1987).

Plasmids Exo1, Exo3, Exo4, Exo5, Exo7, Exo8, and Exo12 containing the *Bcl*I–*Bam*HI fragment from SV40 were obtained from David Bentley and Mark Groudine (Bentley & Groudine, 1988).

DNA Templates. The templates used for transcription were generated by restriction digestion of the plasmid at a unique site followed by addition of single-stranded polydeoxycytidylate extensions of 30–100 residues using terminal deoxynucleotidyl transferase (Kadesch & Chamberlin, 1982). The template with two single-stranded polydeoxycytidylate extensions was digested at additional sites in order to limit transcription to the sequences of interest and to prevent transcription in the opposite orientation.

Transcription Reactions. Transcription was carried out as described previously (Kerppola & Kane, 1988). Standard reaction conditions were 70 mM Tris-HCl (pH 8.0), 150 mM NH_4Cl, 20% (v/v) glycerol, 6 mM $MgCl_2$, 5 mM spermidine, 1 mM dithiothreitol, and nucleotides as indicated at 37 °C. Purified calf thymus RNA polymerase II was used at 1–5 μg/mL in the initiation phase. Template DNA was present at a 2–10-fold molar excess over active RNA polymerase molecules. Transcription under high nucleotide concentrations was initiated with 800 μM each of ATP, UTP, and GTP, and 20 μM [α-^{32}P]CTP (1 μCi/20 pmol). After 1 min 45 s, 9 volumes of chase buffer containing 800 μM each of ATP, UTP, and GTP, 100 μM unlabeled CTP, and 100 μg of heparin/mL was added (end-label conditions). Transcripts synthesized under these conditions are labeled primarily at the 5′ end, and the molar abundance of such transcripts is directly proportional to the amount of radioactivity in electrophoret-

ically separated transcripts. Transcription under low nucleotide concentrations was initiated with 800 μM GTP and 20 μM each of ATP, UTP, and [α-^{32}P]CTP (1 μCi/20 pmol). After 1 min 45 s, 9 volumes of chase buffer with 100 μg of heparin/mL containing no nucleotides was added, reducing nucleotide concentrations to 80 μM GTP and 2 μM each of ATP, UTP, and CTP. Transcripts synthesized under these conditions are uniformly labeled throughout the transcript, and the densitometric scans were corrected for the number of C residues in the transcript in order to obtain the relative molar abundance of such transcripts. Samples were withdrawn from the transcription reactions at various times and stopped by the addition of an equal volume of a solution containing 200 mM Tris (pH 7.5), 50 mM EDTA, 600 mM NaCl, 2% sodium dodecyl sulfate, 100 μg of proteinase K/mL, and 200 μg of tRNA/mL and incubated for 10 min at room temperature. Nucleic acids were precipitated twice with ethanol, denatured in 80% (v/v) formamide at 90 °C for 5 min, and separated on denaturing polyacrylamide gels. Gels were dried and exposed to XAR-5 film with an intensifying screen at −80 °C. Termination efficiency was quantitated by densitometric scanning of the autoradiograms. Marker RNAs of known molecular weights were prepared by transcription of plasmids pKK5-1 (Brosius, 1984) and pKK34-121 (Thayer & Brosius, 1985) with *E. coli* RNA polymerase.

Transcript Release. To determine if the nascent transcript was released at sites of transcriptional stopping, nitrocellulose filter binding was performed by a modification of the procedure by Dedrick et al. (1987). Nitrocellulose filters (Schleicher & Schuell, BA85) were presoaked in a solution of boiling 70 mM Tris-HCl (pH 8.0) and were rinsed several times with filter rinse solution [70 mM Tris-HCl (pH 8.0), 50 μg/mL heparin, and 10 μg/mL poly(rC)] immediately prior to use. Transcription was stopped after 20 min of elongation by the addition of α-amanitin to 1 μg/mL, and a 100-μL aliquot was diluted with 100 μL of quench solution [70 mM Tris-HCl (pH 8.0) and 20 μg/mL poly(rC) and NaCl as indicated]. Samples were either filtered immediately or filtered after incubation at 37 °C as indicated. Half of the sample was passed through a nitrocellulose filter at a flow rate of 0.2 mL/min. The other half was processed as the total reaction (T). The filter was washed with 100 μL of Filter Rinse solution. The filtered transcription reaction and the wash were collected and are referred to as the filtrate (F). The filter-bound RNA was eluted with 400 μL of 100 mM Tris (pH 7.5), 25 mM EDTA, 300 mM NaCl, 1% sodium dodecyl sulfate, 50 μg of proteinase K/mL, and 100 μg of tRNA/mL for 60 min at 37 °C. This eluted RNA represents that which is bound (B) to nitrocellulose as a ternary complex. The total reaction was adjusted to 200 μL by addition of 100 μL of transcription buffer, and 200 μL of stop solution containing 200 mM Tris (pH 7.5), 50 mM EDTA, 600 mM NaCl, 2% sodium dodecyl sulfate, 100 μg of proteinase K/mL, and 200 μg of tRNA/mL was added to the total and filtrate. Nucleic acids were precipitated with ethanol and analyzed by gel electrophoresis.

Mobility Analysis. To investigate the electrophoretic mobilities of restriction fragments containing termination sites, 1 μg of plasmid DNA was digested with the restriction endonucleases indicated. Digestion was stopped by the addition of 0.1 volume of 30% glycerol, 0.1 M EDTA, 1% SDS, and 0.25% xylene cyanol. In control experiments, the restriction fragments were first isolated from the digestion reaction by phenol/chloroform extraction and ethanol precipitation. The samples were heated to 65 °C for 10 min, and the restriction fragments were separated on 8% polyacrylamide [7.8% acrylamide and 0.2% *N*,*N*′-methylenebis(acrylamide)] gels in 89 mM Tris–borate/2 mM EDTA, pH 8.0, buffer at the temperature indicated. The gels were run at a field strength of 10 V/cm until xylene cyanol had migrated 21 cm (8–16 h) and were stained with ethidium bromide. The apparent sizes of the terminator-containing fragments were determined from the best fit (Duggelby et al., 1981) to the migration of marker fragments generated by digestion of plasmid pUC18 (Yanisch-Perron et al., 1985) with *Hae*III and *Dra*I, and plasmid pIBI76 (provided by International Biotechnologies, Inc., New Haven, CT) with *Hae*III or *Hin*fI. Some of the marker fragments displayed variable migration at different temperatures. Therefore, only those marker fragments that displayed similar relative mobilities at different temperatures were used in the analysis.

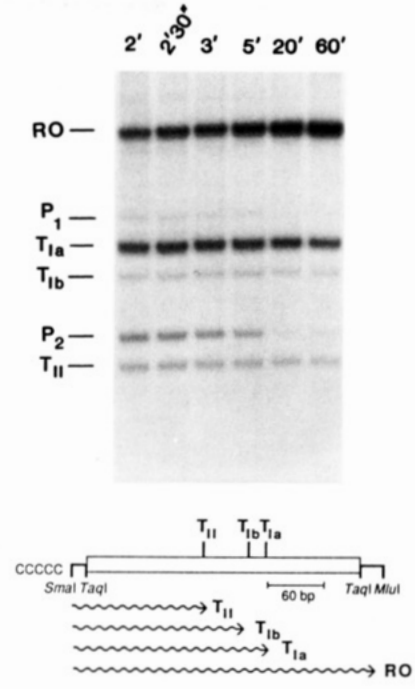

FIGURE 1: Time course of RNA polymerase II transcription elongation across H3.3 gene sequences. The template described below was transcribed with purified RNA polymerase II under end-label conditions with high nucleotide concentrations. Aliquots were removed from the transcription reaction at the times indicated above the lanes, and the transcripts were separated on a denaturing polyacrylamide gel. The positions of transcripts terminated at the T_{Ia}, T_{Ib}, and T_{II} sites, paused at the P_1 and P_2 sites, as well as run-off transcripts (RO) are indicated. The sizes of the transcripts were determined by comparison with RNA size markers (not shown). The template used for transcription was generated by adding 3′-poly(deoxycytidylate) extensions (Kadesch & Chamberlin, 1982) at the *Sma*I site of plasmid pTK242A. The template was digested with *Mlu*I and *Sst*I to limit transcription to the sequences of interest and to prevent transcription in the opposite orientation. The open box indicates H3.3 intron 1 sequences, and the lines indicate vector sequences. The positions of the previously identified H3.3 T_{Ia}, T_{Ib}, and T_{II} termination sites (Reines et al., 1987) are indicated.

RESULTS

An Orientation-Dependent Signal for Transcription Termination in the First Intron of the Human H3.3 Gene. The intrinsic termination sites in the first intron of the human H3.3 gene (Reines et al., 1987) were subcloned downstream of a unique *Sma*I site in plasmid pTK202. Transcription across these sequences on a linearized, 3′-extended template by purified RNA polymerase II generates three transcripts that persist after 1 h of elongation (Figure 1). The sizes of these transcripts are consistent with termination at the previously mapped T_{Ia}, T_{Ib}, and T_{II} sites (Reines et al., 1987). Two

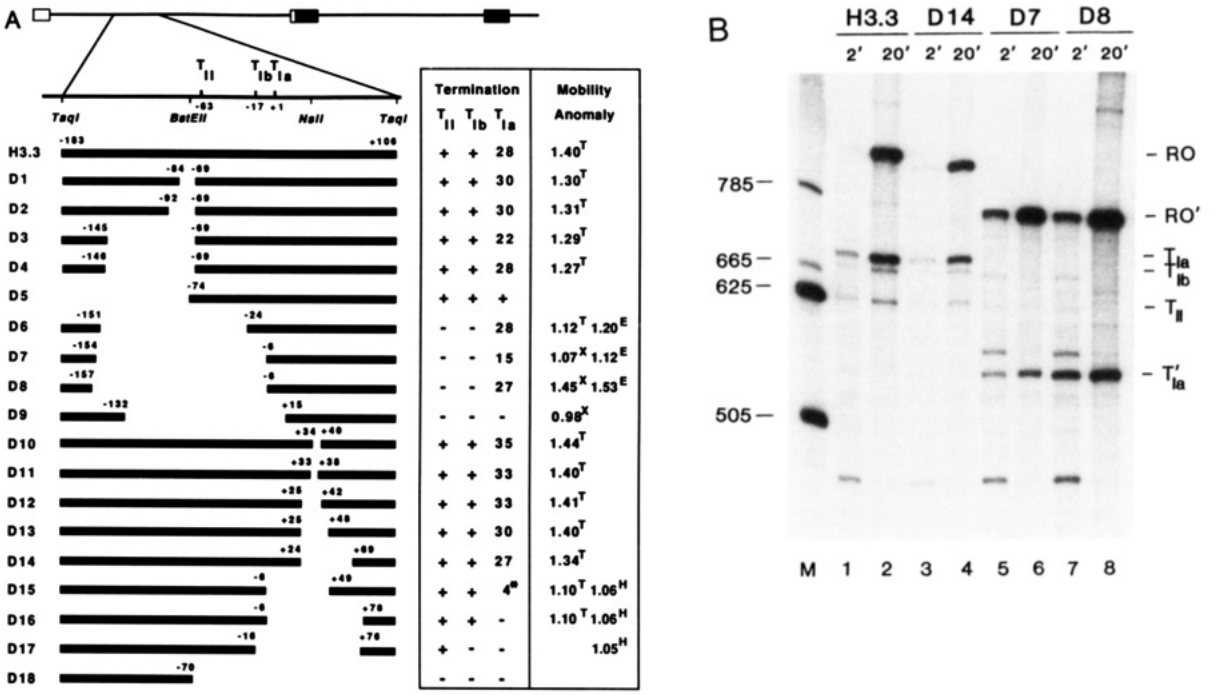

FIGURE 2: Effect of deletion of flanking sequences on transcription termination at the H3.3 termination sites. (A) Templates deleted for sequences flanking the H3.3 termination sites. Deletion derivatives of the H3.3 termination region were generated by limited *Bal*31 nuclease digestion. Templates for transcription with purified RNA polymerase II were generated as in Figure 1, except that the 3′ extension was added at a *Bsu*36 I site 476 bp upstream of the H3.3 sequences. The deletion end points are indicated relative to the first T residue of the first T run at the T_{Ia} site which is numbered +1 (Table I). The preceding residue is numbered −1. Termination efficiency was quantitated for T_{Ia} by densitometric scanning of the autoradiograms; the efficiency is expressed as the percent of the enzyme which stops at this site. A (+) indicates termination with the same efficiency as for the parent template, and a (−) indicates that no termination was detected. An asterisk indicates that a new termination site was created by the deletion through fusing sequences that do not cause termination in their native context. The mobility anomaly for restriction fragments which contain the termination region approximately in the center is indicated. The restriction fragments analyzed were between 204 and 303 bp in size and are indicated with superscripts: *Taq*I(T), *Eco*O109 (E), *Hin*cII–*Xho*I (X), *Hin*fI (H). (B) Transcription of H3.3 templates deleted for sequences flanking the termination sites. The templates indicated above the lanes were transcribed with purified RNA polymerase II. Transcription was under the same conditions as in Figure 1, and samples were withdrawn from the reaction at the times indicated above the lanes. The terminated (T_{Ia}, T_{Ib}, T_{II}, T_{Ia}') and run-off (RO, RO′) transcripts are indicated. Lane M contains RNA size markers.

transcripts appear transiently and represent sites of transcriptional pausing we designate P_1 and P_2. Since the efficiency of transcription termination on this template is the same as that observed for a template containing additional 5′ and 3′ sequences from the H3.3 gene (Reines et al., 1987), all signals required for transcription termination are present on the subcloned fragment. These sequences do not cause transcription termination when they are transcribed in the opposite orientation (data not shown). Thus, there is a polarity in the recognition of the termination signal by purified RNA polymerase II.

Effect of Deletion of Flanking Sequences on Transcription Termination at the H3.3 Termination Sites. In order to determine the specific sequences required for transcription termination in the H3.3 gene, we prepared a set of templates deleted for sequences flanking the termination sites (Figure 2A). We have concentrated our analysis on the T_{Ia} site since it is the strongest termination site in this region. RNA polymerase II terminates transcription at the T_{Ia} site when sequences are deleted to position −6 upstream (Figure 2A, D7 and D8; Figure 2B, lanes 5–8) or to position +24 downstream (Figure 2A, D14; Figure 2B, lanes 3 and 4). The termination efficiency is, however, affected by sequences upstream of position −6 since two derivatives with deletion end points at −6, but with different upstream flanking sequences, have different termination efficiencies (Figure 2A, D7 and D8; Figure 2B, lanes 5–8). Deletion of the T-rich sequence at the termination site completely eliminates termination (Figure 2A, D9 and D16). The deletion of sequences surrounding the weaker T_{Ib} and T_{II} termination sites yields similar results (Figure 2A, D4–D6 and D15–D17). Thus, the T-rich sequence is apparently necessary for termination. While little flanking sequence is required for termination at the H3.3 T_{Ia} site (Table I), additional sequences from this gene can affect the efficiency of termination. Also, deletion constructs bring in new flanking sequences which themselves may influence termination efficiency. Thus, we initially examined in detail the minimal sequences needed to signal termination.

Analysis of the Minimal Termination Signal at the H3.3 T_{Ia} Site. In order to identify the minimal termination signal for purified RNA polymerase II, synthetic oligonucleotides containing H3.3 sequences from the T_{Ia} site were inserted into heterologous sequence contexts and tested for their abilities to terminate transcription (Table I). Templates containing oligonucleotide sequences were transcribed under both high and low nucleotide concentrations to allow more quantitative comparisons between termination signals whose termination efficiencies varied over a 100-fold range (for specific conditions, see Figures 1 and 3). Oligonucleotides that contain the two runs of T residues with either upstream or downstream flanking sequences (Table I, O1, O2, and O4) cause transcription termination (Figure 3, lanes 1, 2, and 4), suggesting that the sequences flanking the two T runs are not required for termination. However, an oligonucleotide that contains only the first T run (Table I, O3) terminates transcription inefficiently (Figure 3, lane 3), indicating that sequences downstream of the first T run are part of the termination signal at the T_{Ia} site. These same oligonucleotides were transcribed

Table I: Transcription Termination at Oligonucleotides Containing H3.3 T_{Ia} and c-*myc* T_{II} Sequences

Template[a]	Sequence[b]	Termination Efficiency[c] High NTP	Termination Efficiency[c] Low NTP	Mobility[e] Anomaly
	D7, D8 ↓↓↓↓ D14			
H3.3	ATTTTTAAAAGAGGGACGTTTTTTTCCCTTTTTTGGAGAGGCGGAAACTTGATG	27.9	93	1.39
O1 ^{B1S}/O1 ^{B2S}	GGGACGTTTTTTTCCCTTTTTTGGAGAGGC	9.7/ 9.6	86/85	1.13/1.31
O2 ^{B1S}/O2 ^{B2S}	GGGACGTTTTTTTCCCTTTTTT	13.8/14.3	83/86	1.10/1.27
O3 ^{B1S}/O3 ^{B2S}	GGGACGTTTTTTT	3.8/ 1.6	18/24	0.98/1.07
O4 ^{B1S}/O4 ^{B2S}	TTTTTTTCCCTTTTTTGGAGAGGC	21.9/20.6	94/96	1.30/1.08
O6 ^{XXh}	TTTTTTTCCCTTTTTT	5.4	83	1.16
O8 ^{XXh}	TTTTTTTCCCTTGTTT	3.2	57	1.07
O9 ^{XXh}	TTTGTTTCCCTTGTTT	0.3	15	1.04
O10 ^{XXh}	TTTCTTTCCCTTGTTT	0.4	11	1.05
O11 ^{XXh}	TTTTTTTCCC	2.3	41	1.08
O6O6′ ^{XXh} [d]	TTTTTTTCCCTTTTTTCTCGAGAAAAAAGGGAAAAAAA	8.4	87	1.31
O7O7′ ^{XXh} [d]	TTTGTTTCCCTTTTTTCTCGAGAAAAAAGGGAAACAAA	0.5	32	1.13
	↓↓↓↓↓↓			
MYC	CACTTGCCTTTTAATTTATTTTTTTTATCACTTTAATGCTGAGATGAGTCGAATG	7.6	91	1.21
M1 ^{XXh}	TTTTAATTTATTTTTTTATCACTTT	9.9	87	1.09
M2 ^{XXh}	TTTTTTTATCACTTT	4.6	85	1.09

[a] Templates for transcription were generated as in Figure 1, except that the 3′ extension was added at a *Bsu*36I site between 380 and 469 bp upstream of the sites of oligonucleotide insertion. Oligonucleotides O1, O2, O3, and O4 were tested in two separate sequence contexts (indicated with superscripts B1S and B2S). [b] The sequences of the oligonucleotide inserts and the native H3.3 and c-*myc* genes are shown. The closest deletion end points examined (see Figure 2A) are indicated in the H3.3 sequence. The minimal termination signals defined by the oligonucleotides are underlined in the H3.3 and c-*myc* sequences. The previously mapped sites of transcription termination at the H3.3 T_{Ia} site (Reines et al., 1987) and the c-*myc* T_{II} site (Kerppola & Kane, 1988) are indicated with arrows above the sequences. [c] Termination efficiency was quantitated by scanning densitometry of the autoradiograms. The average standard deviation for the termination efficiencies at high nucleotide concentrations was 1.4% (three experiments) and at low nucleotide concentrations was 4.7% (three experiments). [d] The two oligonucleotides O6O6′ ^{XXh} and O7O7′ ^{XXh} caused termination at additional sites downstream of the T_{Ia} site. [e] The mobility anomaly of an *Apa*LI–*Stu*I fragment which contains the inserted oligonucleotides approximately in the center is indicated. This fragment is 312 bp in size in the O6 ^{XXh}, O8 ^{XXh}, O9 ^{XXh}, and O10 ^{XXh} constructs. The mobility anomaly is expressed as the ratio between the apparent size based on electrophoretic mobility at 4 °C and the true size based on nucleotide sequence. The average standard deviation for the mobility anomalies was 0.01 (three experiments).

in two plasmid templates; the termination efficiencies were the same in both sequence contexts (Table I, compare B1S and B2S templates). Thus, there are no additional sequence elements required for RNA polymerase II termination at this site. These results implicate the sequence TTTTTTTCCCTTTTTT as a minimal signal for RNA polymerase II transcription termination with termination occurring only in the first run of T residues.

In order to test if this minimal signal was sufficient to cause termination, and to analyze the requirement for the two runs of T residues in this signal, synthetic oligonucleotides containing this minimal signal (Table I, O6 ^{XXh}, O6O6′ ^{XXh}), or signals with substitution or deletion mutations (Table I, O8 ^{XXh}, O9 ^{XXh}, O10 ^{XXh}, O11 ^{XXh}, O7O7′ ^{XXh}), were transcribed. While RNA polymerase II does terminate transcription at the minimal signal (Figure 3, lane 5), it efficiently transcribes through signals with single- or double-nucleotide substitutions that interrupt either one or both T runs (Figure 3, lanes 6–9). Therefore, both runs of T residues are required for termination. Oligonucleotides O6 ^{XXh}, O8 ^{XXh}, O9 ^{XXh}, and O10 ^{XXh} (Table I) are the same length and in identical sequence contexts. Thus, effects on the termination reaction can be attributed directly to these sequences. The minimal termination signal transcribed in the inverted orientation does not cause termination, confirming that this signal is orientation-dependent (data not shown).

The results from these experiments indicate that sequences downstream of the first T run are required for RNA polymerase II transcription termination. Since transcription terminates only within the first T run at the H3.3 T_{Ia} site (Reines et al., 1987), RNA polymerase II recognizes at least part of this termination signal at the DNA level.

Analysis of the Minimal Termination Signal at the c-myc T_{II} Site. We have previously shown that purified RNA polymerase II terminates transcription in the c-*myc* gene (Kerppola & Kane, 1988) at the same site (T_{II}) that blocks transcript elongation in vivo (Bentley & Groudine, 1988). In order to determine if the termination signal in the c-*myc* gene also is localized to the sequences immediately flanking the T_{II} termination site, synthetic oligonucleotides containing c-*myc* sequences from the T_{II} site were tested for their abilities to terminate transcription (Table I, M1 ^{XXh}, M2 ^{XXh}). Oligonucleotides that contain the sequence TTTTTTTATCACTTT terminate transcription, suggesting that this sequence contains the minimal signal for intrinsic termination at the c-*myc* T_{II} site. As with the H3.3 T_{Ia} site, transcription terminates only within the first run of T residues in this c-*myc* sequence (Kerppola & Kane, 1988). The requirement for the second run of three T residues has not been tested directly. However, since a run of seven T residues alone is not sufficient to cause termination (see, for instance, O11 ^{XXh} above), downstream nontranscribed DNA sequences very likely form part of the

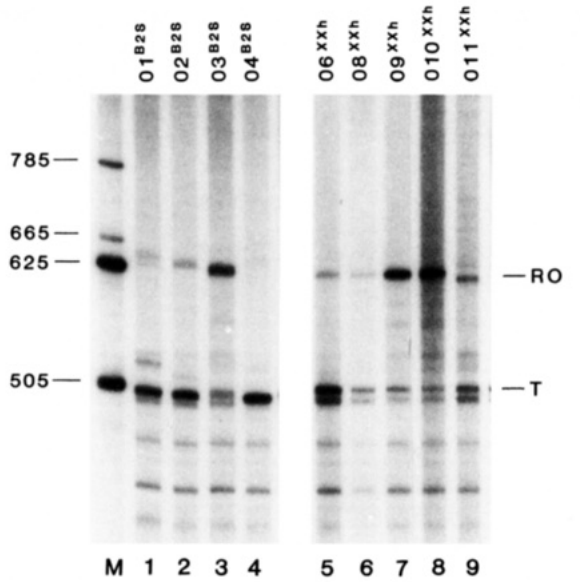

FIGURE 3: Transcription of templates containing H3.3 T_{Ia} oligonucleotides. The templates indicated above the lanes (see Table I) were transcribed with purified RNA polymerase II with low nucleotide concentrations. Samples were withdrawn from the transcription reactions after 60 min of elongation, and the transcripts were separated on a denaturing polyacrylamide gel. The positions of run-off transcripts (RO) and transcripts terminated in the oligonucleotide sequences (T) are indicated. A second termination site is created in upstream vector sequences by insertion of the oligonucleotides in one sequence context (XXh). Lane M contains RNA size markers.

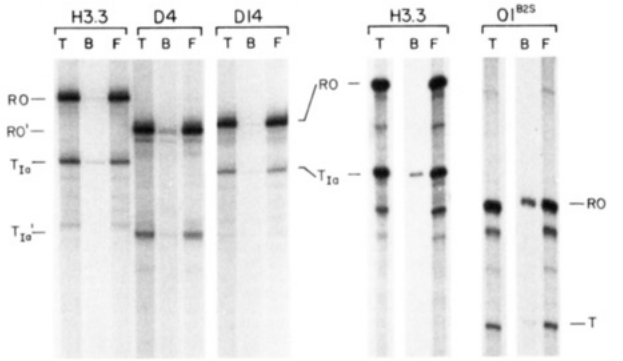

FIGURE 4: Release of the nascent transcripts from deletion and oligonucleotide templates. The templates indicated above the lanes and described in Figure 2 and Table I were transcribed under end-label conditions with high nucleotide concentrations. Transcription reactions were diluted into a quench solution containing a final concentration of 1 M NaCl and incubated for 60 min at 37 °C, and transcript release was determined by nitrocellulose filtration as described under Materials and Methods (T, total; B, bound, F, filtrate). The positions of the run-off (RO) and terminated (T_{Ia}, T) transcripts are indicated.

termination signal also at the c-*myc* T_{II} site.

Release of the Transcript at the Intrinsic Termination Sites. We have shown above that the H3.3 minimal termination signal causes RNA polymerase II to stop elongation. To determine if this signal is also sufficient to effect transcript release, we compared transcript release from the intron sequences tested previously (Reines et al., 1987) with that from the deletion and oligonucleotide derivatives described above using nitrocellulose filter binding (Dedrick et al., 1987). Complete transcript release was observed for all templates tested after 60-min incubation in quench solution containing 1 M NaCl (Figure 4). While transcript release is enhanced by increased NaCl concentration, some release is detected for transcripts produced from all templates even at the lowest salt concentrations used during the filtration (75 mM NH_4Cl) (data not shown). Therefore, the minimal termination signal is sufficient to both stop elongation and cause release of the nascent transcript.

Comparison of Termination Signals in the H3.3 and c-myc Sequences. While the sequences terminating RNA polymerase II transcription at the H3.3 T_{Ia} and the c-*myc* T_{II} sites have two runs of T residues in common, they differ in the exact sequences separating, as well as flanking, these T runs. These sequences are also not strictly conserved at other intrinsic termination sites (Reines et al., 1987; Dedrick et al., 1987; C. Kane, unpublished observations). Indeed, comparison of the sequences at several intrinsic termination sites does not reveal a simple "consensus" termination sequence.

There are several possible explanations for this lack of sequence conservation. The signal may be degenerate, or several completely nonhomologous signals may be recognized. Alternatively, some structural feature not obvious from the primary sequence may act as the signal for transcription termination. Our previous studies have shown that RNA secondary structures are not necessary to stop transcription at these sites (Kerppola & Kane, 1988; Reines et al., 1987; Dedrick et al., 1987). These results are confirmed by the mapping studies described above. The minimal termination signal that is sufficient to cause transcription termination in several different sequence contexts cannot itself form any potential secondary structure. To further check for potential hairpins, we examined the RNA sequences upstream of the termination sites of the deletion and oligonucleotide templates using a computer algorithm (Zuker & Stiegler, 1981) designed to predict RNA secondary structure based on thermodynamic stability (Salser, 1977). No stable ($\Delta G < -3$ kcal) RNA secondary structures could be drawn for the 30 nucleotides upstream of the termination sites for 10 of 14 different transcripts. For transcripts where secondary structures could be drawn, there was an inverse relationship between termination efficiency and the stability of the computer-predicted secondary structure. That is, the higher the termination efficiency, the lower the stability of predicted hairpins (data not shown). Therefore, we conclude that there is neither a requirement for nor a correlation with RNA secondary structure at termination sites for purified RNA polymerase II. Since RNA secondary structure was not implicated in termination, we tested whether DNA structures were important for the termination signal by analysis of some of the structural properties of DNA fragments containing the H3.3 T_{Ia} and c-*myc* T_{II} termination signals.

Restriction Fragment Mobility Analysis Indicates a Sequence-Directed Bend in the H3.3 Termination Region. Since intrinsic termination sites are generally located in T-rich sequences, and since the minimal termination signals at the H3.3 T_{Ia} and the c-*myc* T_{II} sites consist of two runs of T residues centered approximately 10 base pairs apart, we investigated whether the H3.3 termination region might cause the DNA helix to bend. The most commonly used assay for DNA bending is based on the anomalous mobility of DNA fragments during gel electrophoresis (Wu & Crothers, 1984). While other DNA conformations may contribute to this altered mobility, we will operationally associate anomalous gel mobility and DNA bending.

Restriction fragments containing "circularly permuted sequences" (Figure 5A) from the H3.3 termination region were analyzed by polyacrylamide gel electrophoresis (Figure 5B), and the apparent sizes of the fragments were plotted as a function of the site of cleavage within the termination region (Figure 5C). Restriction fragments containing the termination region do migrate anomalously during gel electrophoresis. The mobility anomaly is greatest when the termination region is

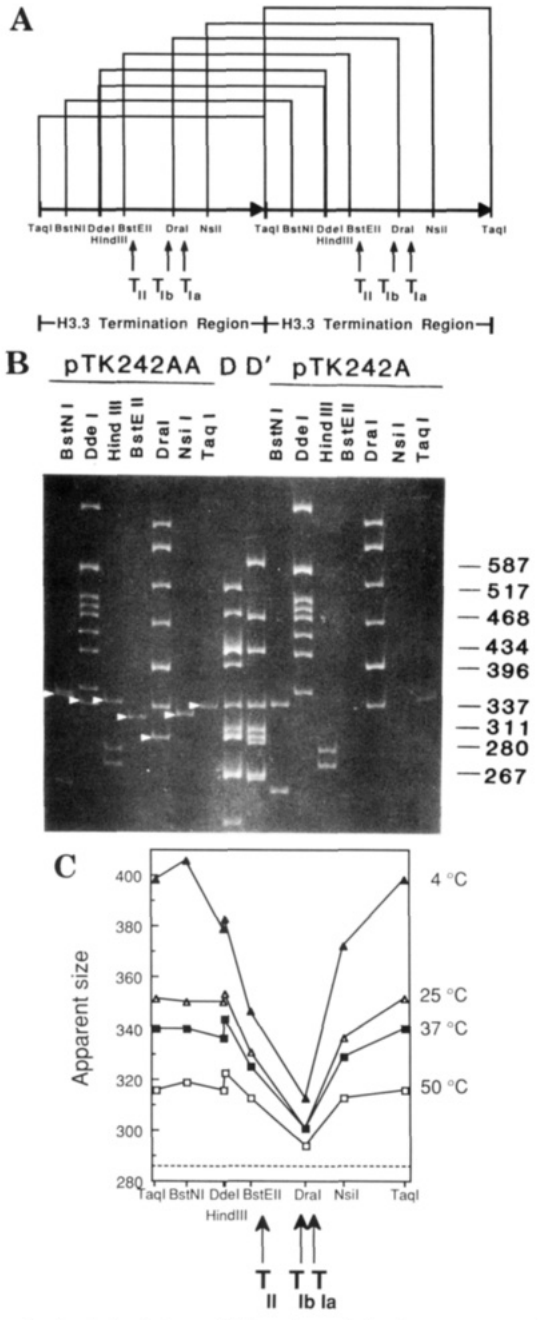

FIGURE 5: Analysis of the mobilities of restriction fragments containing circularly permuted sequences from the H3.3 termination region. (A) Generation of restriction fragments containing circularly permuted H3.3 sequences. Plasmid pTK242AA containing a tandem duplication of the H3.3 termination region was digested with enzymes that cleave at a single site within the fragment. This generates a set of restriction fragments of identical size and sequence composition, but with the termination region located at different positions relative to the ends of the fragments. (B) Polyacrylamide gel electrophoresis of circularly permuted fragments. The restriction fragments were separated on native polyacrylamide gels run at 4, 25, 37, or 50 °C. A gel run at 37 °C is shown in the figure. The fragments containing circularly permuted H3.3 sequences were identified by comparison between digests of plasmid pTK242AA and plasmid pTK242A and are indicated with small arrowheads in the figure. Lane D contains marker fragments generated by digestion of plasmid pUC18 with *Hae*III and *Dra*I, and plasmid pIBI76 with *Hin*fI. The lane D' contains marker fragments generated by digestion of plasmid pIBI76 with *Hae*III. The sizes of selected marker fragments are indicated. (C) Effect of the position of the termination region on the mobility of the restriction fragment. The apparent sizes of the circularly permuted fragments were plotted as a function of the site of cleavage. The true size of all fragments based on nucleotide sequence is shown as a dashed line at 285 bp. The positions of the T_{Ia}, T_{Ib}, and T_{II} intrinsic termination sites are indicated on the bottom of the figure.

in the center of the fragment and is enhanced by electrophoresis at low temperature. These results are consistent with a bend in the DNA helix (Wu & Crothers, 1984) centered in the H3.3 termination region.

Correlation between Bending of the DNA Template and Intrinsic Termination by RNA Polymerase II. In order to determine the relationship between the specific sequences causing the bend and those stopping RNA polymerase II transcription, we analyzed the mobilities of restriction fragments containing the H3.3 T_{Ia} or the c-*myc* T_{II} synthetic oligonucleotides described above (Table I). Restriction fragments containing the H3.3 minimal termination signal migrate with an anomalous mobility (Table I, $O6^{XXh}$). However, restriction fragments containing mutant signals which function as weaker terminators migrate with mobilities closer to those predicted by the DNA sequence (Table I, $O8^{XXh}$, $O9^{XXh}$, $O10^{XXh}$). For these oligonucleotides, which differ only by single- or double-nucleotide substitutions, there is a direct correlation between the mobility anomaly and the efficiency of transcription termination. Since these oligonucleotides are in an identical sequence context and the restriction fragments are of identical size, the changes in termination efficiency and mobility anomaly can be attributed to the same changes in nucleotide sequence. Consequently, the sequences that cause RNA polymerase II transcription termination are identical with the sequences that cause the bend in the DNA helix.

The mobilities of restriction fragments from the H3.3 deletion derivatives (Figure 2A) were also examined. In these cases, however, correlating mobility anomaly with specific sequences is more difficult. The intrinsic curvature of a DNA fragment is influenced by all sequences on the fragment (Ulanovsky & Trifonov, 1987). The restriction fragments used to analyze mobility anomalies for the deletion derivatives contain different flanking sequences and differ in size. Nonetheless, fragments from plasmids with deletions that do not affect termination have mobility anomalies similar to that of the intact termination region. Deletions that eliminate one or more of the termination sites generally reduce the mobility anomaly (Figure 2A, D6–D7, D15–D17), although one such deletion derivative (Figure 2A, D8) has a mobility anomaly that is comparable to that of the intact termination region. Deletion of all the termination sites (D9) completely eliminates the mobility anomaly.

Likewise, restriction fragments containing the O1, O2, O3, and O4 oligonucleotides in two different sequence contexts have markedly different mobility anomalies (Table I, compare B1S and B2S templates) even though the termination efficiencies are similar in each context. These oligonucleotides are of different lengths as well as positioned in different sequence contexts. Again, the mobility anomaly reflects the overall conformation of the restriction fragment rather than the local structure of inserted oligonucleotides. These results emphasize the need to compare inserted sequences of the same length in the same sequence context as described above for O6, O8, O9, and O10 (Table I). For those oligonucleotides, changes in mobility anomaly can be directly correlated with specific nucleotide changes which also affect termination.

Effect of a DNA Bend in the SV40 Genome on Transcription by Purified RNA Polymerase II. To futher investigate the correlation between transcription termination and DNA bending, we transcribed sequences from the SV40 genome which contain a bend visualized by electron microscopy (Hsieh & Griffith, 1988), but whose intrinsic termination properties were not known. This bend lies in the vicinity of

Table II: Comparison of Intrinsic Sites of Transcription Termination[a]

Site	Sequence	Relative Termination Efficiency
H3.3 T_{Ia}	TTTTTAAAAGAGGGACGTTTTTTTCCCTTTTTTGGAGAGGCGG	+++
H3.3 T_{Ib}	ATTTCAAAAAAGCAAGAATTTTTAAAAGAGGGACGTTTTTTTC	+
H3.3 T_{II}	TTCTCTGGTCACCGACCATATTTTTTCCCCCGTTTCTCTCTTC	+
H3.3 T_a	AGTTCTGAACGTTAGATATTTTTTTTCCATGGGGTCAAAGGTA	+
H3.3 T_b	AAAAAAAAAAAATTTCTCTTCTTCCTGTTATTGGTAGTTCTGA	+
H3.3 T_c	TATGATGGGAAACATTTCATTCTCAAAAAAAAAAAATTTCTCT	+
MYC T_{II}	ACTTGCCTTTTAATTTATTTTTTTATCACTTTAATGCTGAGAT	+
RRNB T_1	GAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAAC	+++
RRNB T_2	CCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTCC	+++
TRY T	TTTTTATTTTTTTTATTTTTTTCATTTATTTATTTTTTTTTGA	++

[a] The sequences of several intrinsic termination sites for RNA polymerase II are shown. The termination sites for RNA polymerase II mapped by S1 nuclease analysis are underlined. H3.3, human histone H3.3 gene (Reines et al., 1987); MYC, human c-*myc* gene (Kerppola & Kane, 1988); RRNB, *E. coli rrn*B gene (Dedrick et al., 1987; TRY, *Trypanosoma brucei* spliced leader (C. Kane, unpublished results).

the polyadenylation sites for the SV40 early transcription unit (Hsieh & Griffith, 1988). However, while a stop for purified RNA polymerase II has been reported in this region (Lavialle et al., 1982), intrinsic termination activity of these specific sequences has not been previously documented. We transcribed templates prepared from several plasmids containing the *Bcl*I–*Bam*HI fragment from the SV40 polyadenylation region [see Materials and Methods (Bentley & Groudine, 1988)] with purified RNA polymerase II. On each template, while more than half of the enzyme elongated the full length of the template, a transcript terminated within the SV40 sequences was also produced (data not shown). The sizes of these terminated transcripts determined by comparison with RNA size markers are consistent with termination at a site about 25 bp upstream of the center of the bend. The sequence contains a run of seven T residues within the predicted DNA bend, and the sizes of the transcripts are consistent with stopping in this sequence.

This T run lies downstream of a cryptic polyadenylation site (Connelly & Manley, 1988) but upstream of the major polyadenylation site for the SV40 early transcription unit (Reddy et al., 1979; Kessler et al., 1986). Within the cell, termination from this transcription unit has been assayed by nuclear run-on experiments, and most of the enzyme continues beyond the major polyadenylation site (Connelly & Manley, 1988) and may terminate in or near replication origin sequences (Grass et al., 1987). We have not investigated the cell's use of the intrinsic terminator we have detected in the polyadenylation region of the SV40 genome, but the site clearly can cause termination by the purified polymerase with a reasonable efficiency. Furthermore, this intrinsic termination site also contains a T run and a bend in the template, elements that we propose have a general function in signaling intrinsic termination.

Discussion

We have identified minimal signals for transcription termination by purified RNA polymerase II that consist of short sequence elements containing two runs of T residues phased one helical turn apart. These minimal termination signals cause the DNA helix to bend both in their native context and when inserted into heterologous sequences. Sequences flanking the minimal termination signals appear to influence termination efficiency and DNA bending in concert. A previously identified bend in the SV40 genome also causes intrinsic termination by RNA polymerase II. On the basis of all these results, we suggest that RNA polymerase II transcription termination is caused in part by a structural element that causes a bend in the DNA helix.

While intrinsic termination sites generally occur in T-rich sequences, not all sites contain T runs phased one helical turn apart (Reines et al., 1987; Dedrick et al., 1987; C. Kane, unpublished results; Table II). Likewise, while DNA bends are most commonly associated with phased runs of T residues, not all DNA bends contain such sequences (Hsieh & Griffith, 1988; Bossi & Smith, 1984). The effect of flanking sequences on the extent of DNA bending is also not completely predictable (Ulanovsky & Trifonov, 1987; Koo et al., 1986). Also, while the termination sites described here lie within the center of a bend in the template, other strong intrinsic termination sites that are associated with a bend [*rrn*BT_1 and T_2 (Dedrick et al., 1987); for sequences, see Table II] do not coincide with the bend center (data not shown). Since restriction fragment mobility analysis reflects the overall conformation of the DNA fragment rather than the structural geometry at any one position, the detailed structure of the DNA at these termination sites remains uncertain. Thus, we cannot yet describe the specific structure recognized by the transcribing enzyme at a termination site, except to say that it may be associated with a bend.

However, a bend is not sufficient to cause transcription termination. The termination signal is orientation-dependent while the sequences containing this signal cause a bend when inserted in either orientation. Therefore, transcription termiantion must require some additional element together with the bend. On the basis of the results presented here and our previous observations (Kerppola & Kane, 1988; Reines et al., 1987; Dedrick et al., 1987), it seems likely that at least part

of this additional element is the run of T residues present on the nontranscribed strand. Previously we have shown that there is apparently a sequence context effect that causes purified RNA polymerase II to distinguish between those T runs which function as intrinsic termination sites and those which do not (Kerppola & Kane, 1988; Reines et al., 1987; Dedrick et al., 1987). Perhaps the context effect that determines whether or not RNA polymerase II terminates at a particular T run is the proper positioning of that T run within a bend in the template.

Since the termination signal appears to contain at least two separate elements, the recognition of this signal may depend on a process involving multiple steps. The bend may cause a steric block to translocation of the enzyme along the DNA. Alternatively, the contacts between the transcribing RNA polymerase and the DNA template may be altered by the bend, or the conformation of the enzyme may change as it transcribes across such sequences.

How such changes would direct the enzyme to stop within a T run as opposed to other sequences is unclear. Since part of the T run is transcribed into RNA, recognition of this part of the signal may occur at either the RNA or the DNA level. Perhaps the enzyme makes altered contacts with the thymidine or adenine residues in the template or requires higher concentrations of UTP for efficient elongation at such sites. The local K_s for nucleotide substrates can vary over 2 orders of magnitude for *E. coli* RNA polymerase (Levin & Chamberlin, 1987). It is possible that template conformation alters the enzyme and influences this variability.

Purified RNA polymerase III also can terminate in runs of T residues (Cozzarelli et al., 1983), and the efficiency of termination is affected by sequences flanking the T run (Bogenhagen & Brown, 1981; Mazabrand et al., 1987). A bend in the template has been suggested as part of the signal (Gottlieb & Steitz, 1989), but a role for DNA structure in this signal has not been reported.

ρ-Independent transcription termination in *E. coli* often occurs in runs of T residues (Platt, 1986). While there is a requirement for an upstream sequence that can form a hairpin structure in the RNA (Platt, 1986), additional upstream sequence elements (R. Reynolds and M. J. Chamberlin, unpublished results) as well as downstream nontranscribed sequences dramatically influence the efficiency of transcription termination (Telesnitsky & Chamberlin, 1989; R. Reynolds and M. J. Chamberlin, unpublished results). Even though there is an effect of nontranscribed sequences, no specific DNA structure has been reported as a part of this signal.

In the cell, conditional transcription termination can control gene expression by using signals that overlap with those recognized by purified RNA polymerase II (Bentley & Groudine, 1986, 1988; Eick & Bornkamm, 1986; Cesarman et al., 1987; Kerppola & Kane, 1988). We have shown here that the termination signal for purified RNA polymerase II appears to contain a DNA structural element. Recognition of this signal could be modulated by alteration of DNA structure in the cell. Such structural changes could be mediated by proteins binding to DNA, by changes in DNA superhelicity, or by association with nucleosomes.

While changes in DNA structure could modulate termination by RNA polymerase II, terminator recognition in the cell also may be regulated by proteins that interact directly with either the polymerase or some other component of the elongation complex. Both the initiation complex and the polyadenylation machinery have been implicated in the termination reaction downstream of transcription units (Hernandez & Weiner, 1986; Neuman de Vegvar et al., 1986; Whitelaw & Proudfoot, 1986; Logan et al., 1987; Connelly & Manley, 1988). Termination at intragenic sites such as those studied here may be regulated by factors such as transcription factor SII (Natori, 1982). This protein can bind to purified RNA polymerase II (Horikoshi et al., 1984; Sopta et al., 1985; Reinberg & Roeder, 1987; Rappaport et al., 1988), and it enables the polymerase to elongate through the histone termination region analyzed here (Reines et al., 1989; D. Reines, L. SivaRaman, T. Kerppola, and C. Kane, unpublished results). Thus, not only protein–nucleic acid, but also protein–protein interactions may regulate transcription termination by RNA polymerase II.

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Registry No. RNA polymerase, 9014-24-8.

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Thermodynamic and Spectroscopic Study of Bulge Loops in Oligoribonucleotides†

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ABSTRACT: Thermodynamic parameters for bulge loops of one to three nucleotides in oligoribonucleotide duplexes have been measured by optical melting. The results indicate bulges B_n of A_n and U_n have similar stabilities in the duplexes, GCG^{B_n}GCG + CGCCGC. The stability increment for a bulge depends on more than its adjacent base pairs. For example, the stability increment for a bulge is affected more than 1 kcal/mol by changing two nonadjacent base pairs or by adding terminal unpaired nucleotides (dangling ends) three base pairs away. Thus a nearest-neighbor approximation for helixes with bulges is oversimplified. Many of the non-self-complementary strands used in this study were observed to form homoduplexes. Such duplexes with GA mismatches were particularly stable.

A bulge loop forms in double-helical RNA when the helix is interrupted by unpaired nucleotides on only one strand. Such structures are known to be important for binding of coat protein to R17 virus (Romaniuk et al., 1987) and of ribosomal protein L18 to ribosomal RNA (Peattie et al., 1981; Christiansen et al., 1985). Bulge loops are also thought to be important for intron splicing (Parker et al., 1987; Schmelzer & Schweyen, 1987) and for feedback regulation of the L10 operon in *Escherichia coli* (Climie & Friesen, 1987). Although their functional significance is often unknown, bulge loops are widespread in accepted secondary structures of many

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