MOLECULAR AND CELLULAR BIOLOGY, Oct. 1988, p. 4389–4394
0270-7306/88/104389-06$02.00/0

Vol. 8, No. 10

Intrinsic Sites of Transcription Termination and Pausing in the c-*myc* Gene

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Received 3 May 1988/Accepted 25 July 1988

We have studied transcription elongation and termination in the human c-*myc* gene. Transcription of c-*myc* gene sequences with purified mammalian RNA polymerase II revealed several sites of transcription termination and pausing in the vicinity of the exon 1-intron 1 junction. This region previously has been shown to block transcription elongation in vivo by nuclear run-on analysis (D. Bentley and M. Groudine, Nature [London] 321:702–706, 1986). These sites were recognized by purified RNA polymerase II, and we therefore designated them intrinsic sites of termination and pausing. Two of these sites cause termination of RNA polymerase III transcription as well. RNA polymerase II terminated transcription in a cluster of seven consecutive T residues in the nontranscribed strand and paused during transcription at three additional sites in this region. The intrinsic sites of transcription termination and pausing described here correspond closely to the 3′ ends of transcripts synthesized in *Xenopus* oocytes injected with plasmids containing the c-*myc* termination region (D. Bentley and M. Groudine, Cell 53:245–256, 1988). This correspondence suggests that the intrinsic recognition of these termination and pause sites by purified RNA polymerase II may play a role in the transcription elongation block observed in vivo.

Synthesis of the primary RNA transcript requires that RNA polymerase initiate, elongate, and terminate transcription. These phases of transcription have been extensively studied in procaryotic organisms, and all three have been shown to function in the regulation of gene expression. In eucaryotic organisms, regulation of transcription initiation is a central mechanism for the control of gene expression. However, recent studies suggest that transcription elongation and termination also play important roles in the regulation of many eucaryotic genes (2, 3, 17, 19, 31).

Nuclear run-on analysis has identified regions in which transcription elongation is blocked within several genes (2, 3, 15, 17, 19, 31, 35, 38, 40). In the human c-*myc* gene, a region near the exon 1-intron 1 junction causes a reduction in transcription of the downstream part of the gene (3, 15, 38). The efficiency of this transcriptional block is enhanced when HL-60 promyelocytic leukemia cells are induced to differentiate. This block may therefore modulate c-*myc* gene expression during cell differentiation (3). Furthermore, some Burkitt's lymphoma cell lines that have deregulated c-*myc* gene expression also have a concentration of mutations in the first exon and intron that correlate with abrogation of the transcriptional block (8). Similar intragenic transcriptional blocks have been observed in the human histone H3.3 gene (46), the human immunoglobulin μ heavy-chain gene (19, 35), the murine c-*myc* gene (40), the murine c-*myb* gene (2), the hamster c-*fos* gene (17), the *Drosophila hsp70* gene (21), the simian virus 40 late region (23, 24, 25, 42), the adenovirus major late transcription unit (16, 18, 33, 39, 41), and the human immunodeficiency virus type 1 long terminal repeat (31). Modulation of transcription elongation and termination is therefore a general mechanism for the regulation of gene expression in eucaryotic organisms.

The resolution of the nuclear run-on method does not allow the identification of specific sites of transcriptional stopping, nor does it allow a distinction to be made between transcription termination and pausing. It is also difficult to exclude the possibility that rapid posttranscriptional processing destabilizes the nascent transcript. For these reasons it seems likely that analysis of the sequences and factors responsible for the regulation of transcription termination will require studies using purified systems. An in vitro transcription system has been described that allows purified RNA polymerase II to initiate transcription at a defined site and to synchronously transcribe across a DNA template so that the elongation and termination properties of the purified enzyme can be studied (28).

In this study we have utilized this in vitro transcription system to define intrinsic sites of transcription termination and pausing in the c-*myc* gene, that is, sites that are recognized by the purified mammalian RNA polymerase II protein. This approach allows a direct comparison of sites that act as terminators in vitro with those implicated in blocking transcription in vivo and should cast light on the signals and factors involved in this level of the regulation of c-*myc* gene expression.

MATERIALS AND METHODS

Enzymes. Calf thymus RNA polymerase II was purified by a modification of the method described by Dedrick and Chamberlin (13). The enzyme as purified by Dedrick and Chamberlin (13) is >90% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Transcription of a sheared calf thymus DNA template as well as 3′-polydeoxycytidylate-extended templates is >99% inhibited by 1 μg of α-amanitin per ml. However, analysis of specific transcription products by polyacrylamide gel electrophoresis revealed that these enzyme preparations contained a small amount of RNA polymerase III activity resistant to 2 μg of α-amanitin per ml. (Residual RNA polymerase III has also been observed [51] in RNA polymerase II preparations purified by the method of Hodo and Blatti [27]). Therefore, this enzyme preparation was passed over a DEAE-Sephadex A-25 column and eluted with a linear gradient of 0.1 to 0.8 M $(NH_4)_2SO_4$. The final preparation had no detectable RNA polymerase III activity when assayed by gel electrophoresis

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of transcripts produced in the presence or absence of 2 μg of α-amanitin per ml.

Escherichia coli RNA polymerase holoenzyme and the core RNA polymerase were provided by K. Arndt and B. Krummel and were purified as described by Gonzales et al. (22). Terminal deoxynucleotidyl transferase was purchased from Ratliff Biochemicals, Los Alamos, N.M. The Klenow fragment of DNA polymerase I was purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Ribonuclease H was partially purified from HeLa cells (29). Ribonuclease A was purchased from Sigma Chemical Co. Deoxyribonuclease I was purchased from Worthington Diagnostics, Freehold, N.J., and contaminating ribonuclease activity was removed by bentonite treatment (34). S1 nuclease was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Restriction endonucleases were purchased from Bethesda Research Laboratories, International Biotechnologies Inc., New Haven, Conn., and New England Biolabs, Inc., Beverly, Mass., and digestions were performed under conditions recommended by the supplier.

DNA templates. Plasmids pmyc41 (genomic clone of the human c-*myc* gene [1]) and pGEMHinf (*Hin*fI fragment from pmyc41 spanning the exon 1-intron 1 junction cloned into the *Bam*HI site of pGEM3 [Promega Biotec, Madison, Wis.]) were obtained from D. Bentley and M. Groudine. 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 to 100 residues by using terminal deoxynucleotidyl transferase (28). The template with two single-stranded extensions was digested at additional sites in order to generate a transcript covering the sequences of interest and to prevent transcription from the opposite strand (46).

Transcription reactions. Transcription was carried out as described by Dedrick and Chamberlin (13) except that NH_4Cl was used in place of NaCl. Standard reaction conditions were 70 mM Tris hydrochloride (pH 8.0), 150 mM NH_4Cl, 20% (vol/vol) glycerol, 6 mM $MgCl_2$, 5 mM spermidine, 0.15 mM dithiothreitol, 800 μM each of ATP, UTP, and GTP, and 100 μM CTP at 37°C. Purified calf thymus RNA polymerase II with between 10 and 30% active molecules was used at 1 to 5 μg/ml, and *E. coli* core RNA polymerase was used at 1 μg/ml. Template DNA was present at a 2- to 10-fold molar excess over active RNA polymerase II molecules. End-labeled transcripts were synthesized by initiating transcription with 20 μM [α-^{32}P]CTP (1 μCi/20 pmol) rather than 100 μM CTP. After 1 min 45 s, 9 volumes of chase buffer containing 100 μM unlabeled CTP and 100 μg of heparin per ml were added. Transcripts synthesized under these conditions are labeled primarily at the 5′ end, and the molar abundance of such transcripts is directly proportional to the autoradiographic intensity of electrophoretically resolved RNAs. Samples were withdrawn from the transcription reactions at various times and stopped by the addition of EDTA to a concentration of 70 mM and tRNA to a concentration of 300 μg/ml. Nucleic acids were extracted with phenol-chloroform, precipitated with ethanol, denatured in 80% (vol/vol) formamide at 90°C for 5 min, and separated on denaturing polyacrylamide gels. Gels were dried and exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen (Cronex Lightning-Plus; E. I. du Pont de Nemours & Co., Wilmington, Del.) at −80°C. Transcription was also monitored by determining trichloroacetic acid-insoluble radioactivity (10). Marker RNAs of known sizes were prepared by transcription of plasmids pKK5-1 and pKK34-121 (7, 49) with *E. coli* RNA polymerase. Small marker RNAs were prepared by transcription of T7D111 (32) with *E. coli* RNA polymerase and a low UTP concentration (0.5 μM).

Transcript displacement. To determine the fraction of transcripts displaced from the template (13, 30), transcription reactions stopped by the addition of 1 μg of α-amanitin per ml were treated either with RNase A at a final concentration of 20 μg/ml or with RNase H at 10 U/ml (29) for 10 min at 37°C. Digestion was 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 per ml, and 400 μg of tRNA per ml, and the reaction was incubated for 10 min at 37°C. The transcripts resistant to RNase A or RNase H digestion were quantitated by determining trichloroacetic acid-insoluble radioactivity (10) and were analyzed by polyacrylamide gel electrophoresis.

S1 nuclease analysis. To prepare the hybridization probe, the *Ava*I-*Sph*I fragment of plasmid pGEMHinf was purified from a preparative agarose gel and end labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dGTP (34). The labeled fragment was denatured, and the single strands were isolated from a native polyacrylamide gel (34). Unlabeled RNA was synthesized under standard conditions, and the stopped reaction was treated with 16 μg of DNase I per ml in the presence of 2 mM $CaCl_2$ to digest the template. The runoff transcript was isolated from a preparative polyacrylamide gel. Hybridization and S1 nuclease digestion conditions were as described by Gilman and Chamberlin (20), except that S1 digestion was carried out for 60 min at 30°C. Sequence markers were generated by chemical cleavage as described by Maxam and Gilbert (36).

RESULTS

In order to study transcription elongation and termination in the human c-*myc* gene, we have used an in vitro transcription system that allows efficient transcription initiation by purified RNA polymerase II in the absence of accessory factors. A double-stranded restriction fragment with a single-stranded polydeoxycytidylate extension serves as an efficient 3′-extended template for specific initiation by purified RNA polymerase II at the single-strand–duplex junction (13, 28). The enzyme transcribes the length of the template to generate a runoff transcript unless sequences within the template cause transcription to terminate. Under conditions in which template is present in excess, all active RNA polymerase molecules will initiate transcription essentially simultaneously, and the progression of the transcription complexes across the DNA template can be monitored by polyacrylamide gel electrophoresis of the nascent transcripts.

Transcription elongation and termination in the c-*myc* gene. Transcription elongation across exon 1 and intron 1 of the human c-*myc* gene was studied by using a 3′-extended template on which transcription initiated between the P1 and P2 promoters and ran off in intron 1 (Fig. 1). Transcription of this template with highly purified RNA polymerase II revealed several blocks to transcription elongation (Fig. 1, lanes 1 to 6). A transcript of 750 nucleotides (RO) is consistent with runoff transcription. Several transcripts appeared transiently and chased to longer products. These represent sites of RNA polymerase II pausing. One of these sites corresponds in size to a site designated T_I by Bentley and Groudine, which is recognized by RNA polymerase II in *Xenopus* oocytes (4). A transcript of 530 nucleotides (T_{II})

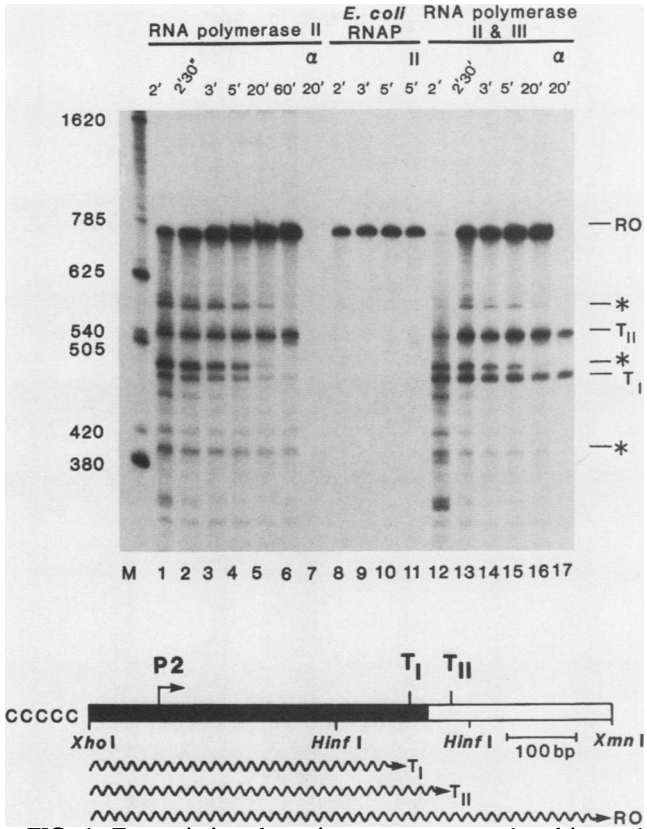

FIG. 1. Transcription elongation on c-*myc* exon 1 and intron 1 sequences. End-labeled transcripts were synthesized with purified RNA polymerase II (lanes 1 to 7), *E. coli* core RNA polymerase (lanes 8 to 11), or partially purified RNA polymerase II containing RNA polymerase III (lanes 12 to 17). Samples were withdrawn from the reactions at the times indicated above the lanes in minutes (') and seconds ("). Lanes 7 and 17, transcription in the presence of 1 μg of α-amanitin per ml. Lane 11, transcripts recovered after a 20-min incubation of purified RNA polymerase II with full-length transcript synthesized by *E. coli* core RNA polymerase. The runoff (RO) and terminated (T_I) and (T_{II}) transcripts are indicated. Paused transcription complexes are indicated with asterisks. Lane M contains molecular weight markers of the indicated sizes. (Numbers to the left of lane M indicate nucleotides.) To generate the template, plasmid pmyc41 was digested with *Xho*I, polydeoxycytidylate extensions were added with terminal transferase, and the template was digested with *Xmn*I and *Not*I. Filled boxes in the diagram at bottom denote exon 1 sequences, and open boxes denote intron 1 sequences.

persisted through the chase. This represents an intrinsic site of transcription termination in the region previously shown to block transcription elongation in isolated human nuclei (3) and in *Xenopus* oocytes (4).

The efficiency of transcription termination at the T_{II} site was approximately 7%, as determined by scanning densitometry. Transcripts terminated at the T_{II} site were released from the transcription complex, as determined by nitrocellulose filter binding (14; data not shown). Thus, they represent true termination products rather than paused complexes.

The terminated and paused transcripts identified in these experiments are primary transcription products of RNA polymerase II and not products of posttranscriptional processing. Transcription was completely inhibited by 2 μg of α-amanitin per ml (Fig. 1, lane 7). In addition, transcription of the template with *E. coli* RNA polymerase yielded only the runoff transcript (Fig. 1, lanes 8 to 10). When this RNA was further incubated with purified RNA polymerase II, there was no degradation to transcripts of the sizes produced by RNA polymerase II (Fig. 1, lane 11).

It has been reported that RNA polymerase III can transcribe c-*myc* sequences in vitro as well as when these sequences are injected into *Xenopus* oocytes (11). Thus, it was of interest to examine the elongation properties of RNA polymerase III in the region of c-*myc* which affects elongation by RNA polymerase II. When the 3'-extended c-*myc* template was transcribed with partially purified calf thymus RNA polymerase II that contained RNA polymerase III (Fig. 1, lanes 12 to 16), terminated transcripts of 470 and 530 nucleotides were observed. The 470-nucleotide transcript corresponds to termination near T_I (which causes RNA polymerase II to pause during transcription), while the 530-nucleotide transcript corresponds to termination near T_{II}. Synthesis of the 470-nucleotide transcript and a fraction of the 530-nucleotide transcript was resistant to 2 μg of α-amanitin per ml (Fig. 1, lane 17) but was sensitive to 100 μg of α-amanitin per ml (data not shown). These terminated transcripts are thus products of calf thymus RNA polymerase III. In addition, when the 3'-extended c-*myc* template was transcribed with partially purified human RNA polymerase III (50), transcription termination near the T_I and T_{II} sites was also observed (data not shown). The efficiency of intrinsic transcription termination by calf thymus RNA polymerase III was 63% at T_I and greater than 98% at T_{II}. Thus, sequences around T_I and T_{II} signal pausing and termination for both polymerases. The termination efficiencies of the two enzymes differed dramatically at these sites, with RNA polymerase III terminating with much higher efficiency. However, since α-amanitin also can affect elongation by RNA polymerase III (S. Scanlon and W. Folk, personal communication), it cannot be ruled out that low concentrations of this inhibitor influence the termination efficiency of this enzyme.

Role of secondary structure in transcription termination. It has been proposed (15) that alternative RNA secondary structures may be involved in the modulation of transcription termination in the c-*myc* gene. We have shown previously that RNA secondary structure is not necessary for the recognition of other intrinsic termination sites by purified RNA polymerase II (14, 46). In order to explore the role of RNA secondary structure in transcription termination in the c-*myc* gene, we have taken advantage of an unusual property of purified RNA polymerase II, in which the enzyme produces transcripts that remain hybridized to the template strand on some templates (13, 28). Thus, the efficiency of RNA polymerase II transcription termination can be compared between templates for which the transcript remains hybridized to the DNA template strand and templates for which the nascent transcript is displaced. The extent of transcript displacement depends on the sequence at the single-strand–duplex junction (13) and ranges from 0 to 80% for the different 3'-extended c-*myc* templates used in these studies. The methods for analyzing transcript displacement and for the direct comparison of transcripts produced by purified RNA polymerase II on both types of templates have been described previously (13, 14, 28, 46; see also Materials and Methods). By determining transcript sensitivity to both RNase A and RNase H, we could distinguish those transcripts which were displaced during transcription from those which were present as an RNA-DNA hybrid. These transcripts were analyzed by gel electrophoresis, and there were

no differences in the termination sites or efficiencies, or the sites of pausing, whether the transcripts were displaced or remained hybridized to the template (data not shown). These results provide evidence against a requirement for RNA secondary structure in pausing or stopping purified RNA polymerase II in these sequences in the c-*myc* gene.

Mapping the sites of transcription termination. To determine the sites of RNA polymerase II and RNA polymerase III transcription termination at the nucleotide level, the 3′ ends of transcripts terminated at T_I and T_{II} were mapped by S1 nuclease protection (Fig. 2). In addition to the fragment protected by the runoff transcript (S1-RO), two clusters of fragments protected by shorter transcripts were observed (S1-T_I and S1-T_{II}) (Fig. 2, lanes 5 and 6). These fragments are not the result of S1 nuclease cleavage at hypersensitive sites within a double-stranded hybrid, since they are absent in reactions with transcripts produced by *E. coli* core RNA polymerase (Fig. 2, lanes 3 and 4) and in reactions with isolated runoff transcripts (Fig. 2, lanes 11 and 12).

The intrinsic RNA polymerase II termination site T_{II} mapped to a sequence of 7 consecutive T residues in the nontranscribed strand (Fig. 3). RNA polymerase III also terminated transcription in this cluster of T residues, as well as in a second cluster 60 base pairs upstream from the T_{II} termination site. The T_{II} intrinsic termination site is located 35 base pairs downstream from the exon 1-intron 1 junction (Fig. 3) in the region that blocks transcription elongation in isolated nuclei (3, 15, 38). Three additional pause sites were located between 45 base pairs upstream and 10 base pairs downstream of the exon 1-intron 1 junction (Fig. 3). The location of these sites was determined by transcription of the template shown in Fig. 2 and comparison of the size of the paused transcripts with small marker RNAs (data not shown). The T_{II} and T_I intrinsic sites of transcription termination and pausing for calf thymus RNA polymerase II correspond closely to the 3′ ends of transcripts synthesized in *Xenopus* oocytes injected with plasmids containing the c-*myc* termination region (4).

DISCUSSION

A conditional block to transcription elongation has been implicated in the regulation of c-*myc* gene expression (3, 15, 38, 40, 43). Mutations in the vicinity of this block can alter c-*myc* gene expression in the cell (8). This region blocks transcription elongation in isolated human nuclei (3, 15, 38) and in *Xenopus* oocytes (4). Furthermore, as we have shown here, this region causes both pausing and termination of transcription by purified calf thymus RNA polymerase II. It is attractive to assume that all of these effects are brought about by common sequence elements that signal termination of transcription by RNA polymerase II and lead to a transcription block in vivo.

Termination within the c-*myc* gene occurs in a cluster of T residues on the nontranscribed strand. Previous studies with purified RNA polymerase II have shown that such T-rich regions are present at every intrinsic termination site for the enzyme, although a run of T residues is not sufficient to cause termination by RNA polymerase II (14, 46). Therefore, additional sequence elements linked to the run of T residues are required to signal transcription termination. Several mutations in c-*myc* genes from Burkitt's lymphoma cells eliminate the block to transcription elongation (8). These mutations suggest that important sequence elements flank the termination sites or that such sequences might affect the function of factors modulating the efficiency of transcription termination.

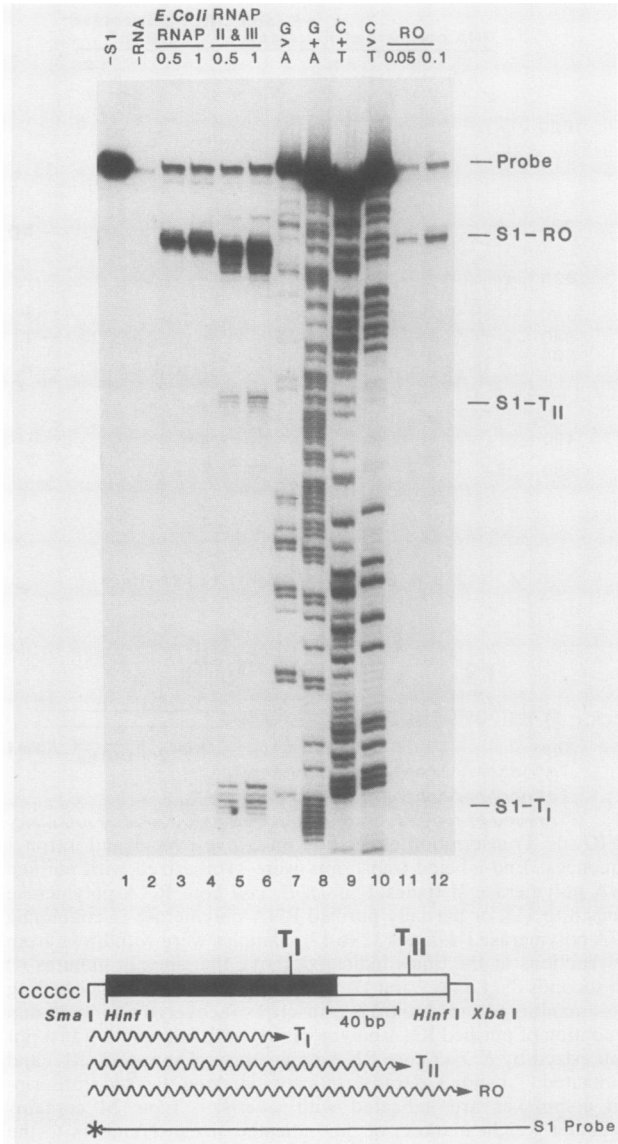

FIG. 2. S1 nuclease mapping of RNA polymerase II and RNA polymerase III termination sites. Transcripts were synthesized with partially purified RNA polymerase II containing small amounts of RNA polymerase III activity. Lanes: 1, undigested probe; 2, probe digested with S1 nuclease in the absence of transcripts; 3 and 4, 0.5- and 1-fmol transcripts synthesized by *E. coli* RNA polymerase; 5 and 6, 0.5- and 1-fmol transcripts synthesized by purified RNA polymerase II containing RNA polymerase III activity; 7 to 10, G > A, G + A, C + T, and C > T sequence markers, respectively; 11 and 12, 0.05- and 0.1-fmol isolated runoff transcripts. Full-length probe (Probe) and fragments protected by runoff (S1-RO) and terminated (S1-T_I, S1-T_{II}) transcripts are indicated. To generate the transcription template, plasmid pGEMHinf was digested with *Sma*I, polydeoxycytidylate extensions were added with terminal transferase, and the template was then digested with *Xba*I and *Eco*RI. Filled boxes in the diagram at bottom denote exon 1 sequences, and open boxes denote intron 1 sequences. The lines represent sequences from the cloning vector.

Expression of the human c-*myc* gene in the cell can be regulated by modulation of the block to transcription elongation at the exon 1-intron 1 junction. In nuclear run-on experiments, the difference in transcription between exon 1 and exon 2 varies from a 3-fold molar excess in undifferen-

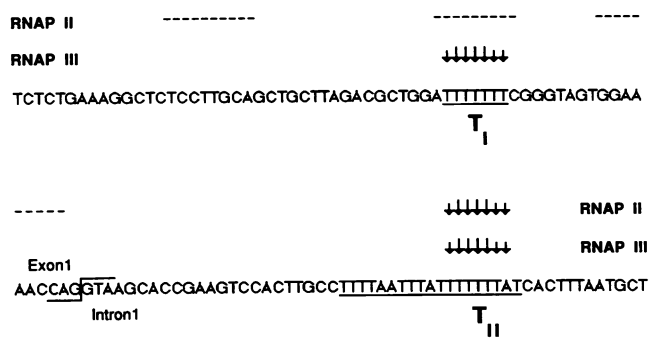

FIG. 3. Sites of transcription termination and pausing by RNA polymerase II and RNA polymerase III in the vicinity of the exon 1-intron 1 junction of the c-*myc* gene. Arrows show the 3′ termini of transcripts mapped by S1 nuclease protection analysis (corrected for the difference in migration between enzymatically and chemically generated fragments). Dashed lines show regions of transcriptional pausing determined on the basis of the migration of paused transcripts synthesized by using the template shown in Fig. 2 relative to that of small marker RNAs of known sizes. The 3′ ends of transcripts synthesized in *Xenopus* oocytes injected with plasmids containing the c-*myc* termination region (4) are underlined in the sequence.

tiated cells to a 15-fold molar excess in differentiated cells (3). Purified RNA polymerase II terminates transcription with 7% efficiency at a site in this region, and it pauses at several additional sites. This low efficiency of intrinsic termination by the purified enzyme suggests that accessory components of the transcription machinery modulate terminator utilization in the cell. It should be noted that there are a number of bacterial rho-independent termination sites that are very efficient in vivo yet are inefficient in vitro with the purified bacterial RNA polymerase in the absence of accessory factors (6, 9, 47).

Studies of the effect of protein synthesis inhibitors on transcription of the c-*myc* gene have been interpreted to indicate the requirement for a labile termination factor (40). Other *trans*-acting factors have been implicated in regulation of elongation in adenovirus (44, 45). Studying the biochemical mechanism of such factors in the regulation of transcription termination is difficult in the absence of a fully defined in vitro system. Such a system was recently utilized for the identification and isolation of a termination factor for vaccinia virus RNA polymerase (48). We are currently investigating the effect of fractionated cell extracts on transcription termination in the c-*myc* gene by purified RNA polymerase II with the goal of characterizing the additional components which regulate terminator recognition in the cell.

The regulatory significance of the finding that RNA polymerase III can also transcribe the early part of the c-*myc* gene (11) is unclear. In the presence of levels of α-amanitin which completely inhibit RNA polymerase II, no cellular transcription of c-*myc* has been detected (3, 15, 38). Likewise, in *Xenopus* oocytes in the presence of 1 to 2 μg of α-amanitin per ml and low DNA template concentrations (conditions under which the 5S gene is readily transcribed), no c-*myc* transcription is detected (4).

However, our results clearly demonstrate that RNA polymerase III initiating transcription on a 3′-extended template recognizes sites close to the exon 1-intron 1 boundary as intrinsic termination sites just as does RNA polymerase III initiating transcription in extracts or in *Xenopus* oocytes (11). RNA polymerase III terminates transcription at two sites coincident with those that block RNA polymerase II transcription elongation. Both sites map within runs of consecutive T residues similar to previously observed sites of termination for purified *Xenopus* RNA polymerase III (12). However, as with RNA polymerase II, T residues alone do not determine the efficiency of intrinsic termination by RNA polymerase III (5, 26, 37; Kerppola and Kane, unpublished data).

ACKNOWLEDGMENTS

We are very grateful for the generosity of David Bentley and Mark Groudine, who provided the c-*myc* plasmids, information prior to publication, and many helpful suggestions. We thank Michael Van Dyke and Robert Roeder for providing human RNA polymerase III. We also thank Daniel Reines, Karen Arndt, and Michael Chamberlin for critical reading of the manuscript.

This work was supported in part by a grant from the National Institutes of Health and in part by a University of California Predoctoral Fellowship to T.K.K.

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