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Transcription Elongation in the Human c-*myc* Gene Is Governed by Overall Transcription Initiation Levels in *Xenopus* Oocytes

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Both transcription initiation and transcription elongation contribute to the regulation of steady-state c-*myc* RNA levels. We have used the *Xenopus* oocyte transcription assay to study premature transcription termination which occurs in the first exon and intron of the human c-*myc* gene. Previous studies showed that after injection into *Xenopus* oocytes transcription from the c-*myc* P1 promoter resulted in read-through transcripts whereas transcription from the stronger P2 promoter resulted in a combination of prematurely terminated and read-through transcripts. We now demonstrate that this promoter-specific processivity results from the overall amount of RNA polymerase II transcription occurring from either promoter. Parameters that reduce the amount of transcription from P1 or P2, such as decreased concentration of template injected or decreased incubation time, result in a reduction in the ratio of terminated to read-through c-*myc* transcripts. Conversely, when transcription levels are increased by higher concentrations of injected template, increased incubation time, or coinjection with competing template, the ratio of terminated to read-through transcripts increases. We hypothesize that an RNA polymerase II processivity function is depleted above a threshold level of transcription initiation, resulting in high levels of premature transcription termination. These findings account for the promoter-specific effects on transcription elongation previously seen in this assay system and suggest a mechanism whereby limiting transcription elongation factors may contribute to transcription regulation in other eukaryotic cells.

The importance of transcription elongation as a mechanism of eukaryotic gene regulation is becoming increasingly apparent. Examples of RNA polymerase II genes which are controlled at the level of transcription elongation include proto-oncogenes c-*myc*, c-*fos*, c-*myb*, L-*myc*, and N-*myc* (2, 12, 26, 36, 37, 57), the human adenosine deaminase gene (10), the epidermal growth factor receptor gene (14), the β-globin gene (30), the *Drosophila hsp70* gene (28, 46), and a number of genes of viruses such as the human immunodeficiency virus (HIV) (22, 27), adenovirus (25), simian virus 40 (43), and polyomavirus (50). For reviews, see references 23 and 53. In many of these examples, transcription initiation levels remain constant, with RNA polymerase II transcription complexes being regulated to either arrest or elongate through specific pause sites located within the first few hundred base pairs of the transcription unit.

An example of the regulation of transcription through release of an arrested polymerase is that of the human c-*myc* gene. The human c-*myc* gene is regulated by a block to transcription elongation during a number of in vivo processes such as differentiation of HL-60 cells and stimulation of quiescent cells by mitogens (2, 29). In addition, the block to transcription elongation is lost in several tumor cells, such as HeLa and Burkitt's lymphoma cells, contributing to high or constitutive levels of c-*myc* mRNA in these cells (52, 54). Nuclear runoff transcription assays have located the site of the transcription block to within the first few hundred base pairs of the human c-*myc* gene, a region encompassing the 3′ end of exon 1 and 5′ end of intron 1.

The molecular mechanisms that regulate eukaryotic transcription elongation are not fully understood but are thought to involve the action of transcription elongation factors that interact with or modify the RNA polymerase II transcription machinery (23).

Paradigms for such action are provided by the N and Q antitermination functions of bacteriophage lambda. The N and Q proteins interact with RNA polymerase to alter its transcription elongation properties, leading to expression of genes located downstream of termination sites (13, 32, 44).

Several transcription elongation factors for RNA polymerase II have been identified. TFIIS (S-II, DmS-II) suppresses pausing and is thought to interact transiently with RNA polymerase II itself while the polymerase is at a pause site (1, 20, 42, 51). Some recent data suggest that TFIIS may achieve this read-through function by conferring a 3′-to-5′ exonuclease capacity to RNA polymerase II (21, 41, 56). TFIIF (Rap30/74, factor 5) affects transcription elongation by stimulating the transcription rate (1, 20). TFIIF is also an essential transcription initiation factor, entering into the preinitiation complex at the promoter (1, 4, 5, 39). Two other factors, TFIIX from HeLa cells and YES from yeast cells, enhance transcription elongation by affecting the elongation rate (1, 6, 20). A positive transcription elongation factor (P-TEF) has been identified in *Drosophila* transcription extracts (31). The presence of P-TEF converts paused transcription elongation complexes to processive ones. This factor is present in limiting amounts in transcription extracts and acts early in the transcription process, i.e., subsequent to transcription initiation and prior to elongation past 500 bases. These features, plus its sensitivity to the nucleotide analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), distinguish P-TEF from the previously mentioned transcription elongation factors.

The only promoter-specific RNA polymerase II transcription elongation factor identified to date is the Tat protein of HIV. The HIV-encoded Tat protein increases the levels of HIV type 1 mRNA synthesis by interacting directly or indirectly at the TAR sequence, a stem-loop structure in the

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HIV mRNA, located immediately 3′ to the transcription initiation site (47, 48). Nuclear runoff transcription assays and in vitro analyses show that Tat positively affects RNA polymerase II processivity and may also increase levels of transcription initiation (22, 27).

A widely used system for the study of RNA polymerase II transcription elongation is the *Xenopus* oocyte transcription assay. This assay has been used to study transcription arrest from the human c-*myc* gene (3, 33, 54), the murine c-*myc* gene (45, 59), the human and mouse adenosine deaminase genes (8, 9, 40), and the *Xenopus* α-tubulin gene (34).

The *Xenopus* oocyte transcription assay was initially used to study premature transcription termination from the human c-*myc* gene for two reasons. Firstly, precise mapping of c-*myc* pause or premature-termination sites is not possible in nuclear runoff transcription assays because of the necessity for the use of long probes. Secondly, prematurely terminated exon 1 transcripts were not detectable in mammalian cells, presumably because short, nonpolyadenylated RNAs are highly unstable. Therefore, in order to study the pause or termination sites, cloned c-*myc* genes were injected into the germinal vesicles of *Xenopus* oocytes, in which nonpolyadenylated RNAs are stable. In *Xenopus* oocytes, transcription accurately initiates at the c-*myc* P1 and P2 promoters and terminates at a number of sites near the 3′ end of exon 1 (3). The 3′ ends of these short c-*myc* RNAs are believed to correspond to the sites of pausing or premature termination in vivo and correspond to several intrinsic termination sites mapped in vitro by using purified RNA polymerase II (24). These sites include two thymidine-rich regions near the exon 1-intron 1 splice junction as well as a number of other discrete sites within the 3′ half of the first exon.

Use of the *Xenopus* oocyte system led to two conclusions regarding the human c-*myc* block to transcription elongation. (i) A 95-bp region near the end of exon 1 is sufficient to obtain a block to transcription elongation within or near this sequence (3). (ii) Promoter-specific effects on the efficiency of this transcription block may exist. Transcription initiating at the c-*myc* P2 promoter or the herpes simplex virus thymidine kinase (TK) promoter results in an efficient block to transcription elongation, whereas transcription from the c-*myc* P1 promoter or the adenovirus major late promoter results in a less efficient block to transcription elongation (3, 54).

We now report that the previously observed promoter effects on transcription elongation efficiency may be determined by the overall levels of RNA polymerase II transcription occurring in this assay system rather than being related to any direct elongation property of the promoter. The efficiency of transcription elongation appears to be controlled by a transcription elongation function, present in limiting amounts in *Xenopus* oocytes. This elongation function is titrated by high template concentrations, by increased transcription over time, or by competing transcription from a heterologous promoter. These results have general implications for the use of the *Xenopus* oocyte transcription assay in studies of transcription elongation and suggest that limiting transcription elongation factors may provide one mechanism for control of transcription elongation in other eukaryotic cells.

MATERIALS AND METHODS

DNA templates. The WT, ΔP1, and ΔP2 plasmids were constructed as described previously (54). Briefly, the 8-kb *Hin*dIII-*Eco*RI fragment of the human c-*myc* gene was cloned into plasmid pVZ1 to create the WT c-*myc* plasmid. The *Hin*dIII-*Eco*RI fragment contains the entire transcribed region of the human c-*myc* gene with 2 kb of upstream DNA and 1 kb of downstream DNA. The ΔP1 plasmid was constructed from WT by deletion of 29 bp constituting the TATA box to cap site of the c-*myc* P1 promoter. The ΔP2 plasmid was constructed by deleting 31 bp constituting the TATA box to cap site of the c-*myc* P2 promoter.

The TK plasmid was constructed by inserting a *Bam*HI fragment constituting the herpes simplex virus TK promoter and including 300 bp of 5′ flanking sequence into the vector, pVZ1 (33).

Plasmid DNA was prepared by either CsCl gradient centrifugation or polyethylene glycol precipitation for use in *Xenopus* oocyte injections. Templates were injected as closed circular DNA.

Oocyte injections. Plasmid DNA was injected into *Xenopus laevis* oocytes, as described previously (3, 54), in 10-nl volumes at concentrations specified in figure legends. After injection, oocytes were incubated at 18°C for the times indicated. Healthy oocytes (10 to 20 per sample) were pooled and homogenized in guanidinium isothiocyanate, and RNA was extracted for S1 analysis. Alternatively, germinal vesicles (30 to 60 per sample) were manually dissected and frozen for nuclear runoff transcription assays.

RNA analyses. RNA was analyzed by S1 nuclease assays using single-stranded, end-labelled probes. The 5′-end-labelled probe was prepared as described previously (54) and is homologous to the ΔP2 RNA. P1 transcripts synthesized from the WT template loop out over the deletion point in the probe but continuously protect the DNA probe through this region. Transcripts initiating at the P1 promoter protect a 401-base probe fragment, and transcripts initiating at the P2 promoter protect a 270-base fragment.

The 3′-end-labelled probe (Nci probe) was prepared by isolating the 609-bp fragment between the *Nci*I sites 66 and 675 bp downstream of the c-*myc* P2 promoter from the wild-type c-*myc* plasmid, pBS2 (54). The ends of the Nci fragment were filled in by using T4 DNA polymerase and radioactively labelled dCTP. The sense strand of the Nci fragment was then removed from the antisense strand by hybrid selecting it onto single-stranded c-*myc* DNA and extending it with Klenow in the presence of deoxynucleoside triphosphates (dNTPs) as follows. The single-stranded DNA was prepared by cloning the 2.3-kb *Kpn-Xba* fragment of c-*myc* into the pVZ1 vector in an orientation that allowed a single-stranded DNA, homologous to the sense strand of the Nci fragment, to be rescued with helper phage. The end-labelled sense Nci strand was hybrid selected by denaturing the end-labelled Nci fragment at 95°C and cooling it slowly to 37°C in the presence of the single-stranded c-*myc* DNA to allow the sense strand to anneal to the single-stranded antisense c-*myc* DNA. The annealed Nci strand was then extended with Klenow, creating a long Nci sense strand which could easily be separated from its Nci antisense strand partner during gel purification. The antisense single-stranded Nci strand was purified from the mixture by electrophoresis on a denaturing polyacrylamide gel by cutting out the 609-base single-stranded fragment and eluting.

The 3′-end-labelled probe (Xho probe) was prepared by isolating the 751-bp fragment extending from the *Xho*I site 95 bp upstream of the c-*myc* P2 promoter to the *Xmn*I site 656 bp downstream of the c-*myc* P2 promoter from plasmid ΔP2. The ends of the *Xho*I-*Xmn*I fragment were filled in by using T4 polymerase and radioactively labelled dCTP and TTP. The antisense strand was purified from the sense strand as

described above for the Nci probe. Transcripts initiating at the P1 promoter and synthesized from the ΔP2 plasmid are homologous to the Xho probe. P1-initiated transcripts synthesized from the WT plasmid form a loop-out over the 31-bp deletion region but fully protect the Xho probe through this region.

The TK probe was prepared by annealing a 32-base oligonucleotide, homologous to a region from 213 to 245 bp downstream of the TK promoter and 5′ end labelled with polynucleotide kinase, to single-stranded DNA prepared from the TK plasmid, as described above. The oligonucleotide was extended in the presence of Klenow and dNTPs, the resulting double-stranded plasmid was cut with *Bam*HI, and the single-stranded fragment extending from the 5′-end label to the *Bam*HI site was purified on a denaturing polyacrylamide gel. Transcripts initiating at the TK promoter protect a 245-base probe fragment.

S1 nuclease analyses were performed as described previously (54). The amount of oocyte RNA assayed was 3 μg per sample unless specified otherwise in figure legends. For quantitation of S1 products, bands were excised from dried gels following autoradiography, immersed in scintillation fluid, and counted in a scintillation counter.

Nuclear runoff transcription assays. *Xenopus* oocyte germinal vesicles (30 to 60 per sample) were manually dissected into oocyte dissection buffer and frozen at −70°C in the nuclear freezing buffer, as described elsewhere (3). Runoff transcription assays were performed with [^{32}P]UTP as the label as described previously (54), and transcription products were hybridized to excess sense and antisense single-stranded probes slot blotted onto GeneScreen Plus filters. The single-stranded M13 probes for human c-*myc* regions were sense and antisense strands of (i) 5′, a 568-base *Nsi*I-*Sma*I fragment extending from −672 to −104 upstream of the human c-*myc* P1 promoter, (ii) Ex 1, a 445-base *Xho*I-*Pvu*II fragment within c-*myc* exon 1 extending from +66 to +511 relative to the P1 promoter, and (iii) Int 1, a 606-base *Sst*I fragment within c-*myc* intron 1 extending from +936 to +1542 relative to the P1 promoter (54). Uridine contents of sense transcripts in these regions were 121, 73, and 136, respectively. As controls for amounts of RNA polymerase II transcription, double-stranded plasmid probes were included. These were pBR322 and a fragment of the chicken histone H2b gene cloned into pBR322 (7).

For quantitation of nuclear runoff transcription assays, autoradiographs were scanned in a Joyce-Loebl chromoscan 3.

RESULTS

Patterns of c-*myc* transcription initiation and termination in *Xenopus* oocytes. In a previous study, we observed differences in the elongation properties of transcription initiating from the two c-*myc* promoters, P1 and P2 (54). In these studies, we used the *Xenopus* oocyte injection system to study the block to transcription elongation that occurs in the first exon and first intron of the human c-*myc* gene. Northern (RNA) analyses of human c-*myc* transcripts synthesized following injection of human c-*myc* plasmids into *Xenopus* oocytes showed that transcription from the c-*myc* P1 promoter resulted in full-length transcripts whereas transcription from the stronger P2 promoter resulted in a combination of short and full-length transcripts. The c-*myc* P1 and P2 promoters are 161 bp apart. The short P2-initiated transcripts were estimated to be between 350 and 500 bases long, consistent with previous studies which mapped the 3′ ends of prematurely terminated P2 transcripts to sites near the 3′ end of c-*myc* exon 1 (3). These previous analyses were performed with a wild-type c-*myc* plasmid containing both promoters and with deletion plasmids in which either the P1 or the P2 promoter was deleted (54). The short exon 1 c-*myc* RNAs did not result from processing but arose from either premature termination or RNA polymerase II pausing (3). Although it has not been determined whether the short exon 1 RNAs are due to premature termination or pausing, in this report we will refer to the short c-*myc* exon 1 RNAs as terminated.

In order to establish the basis for the observed promoter-specific differences in transcription elongation when human c-*myc* genes are transcribed in *Xenopus* oocytes, we first mapped both 5′ and 3′ ends of the resultant transcripts using S1 analysis with end-labelled, single-stranded probes (Fig. 1). The c-*myc* templates injected into *Xenopus* oocytes were (i) WT, an 8-kb fragment of wild-type c-*myc* sequences cloned into vector pVZ1, (ii) ΔP1, the same plasmid as WT but with a 29-bp deletion of the P1 promoter from the TATA box to cap site, and (iii) ΔP2, the same plasmid as WT but with a 31-bp deletion of the P2 promoter from the TATA box to the cap site. When the WT c-*myc* plasmid containing both the P1 and the P2 promoters was injected into *Xenopus* oocytes, transcripts were initiated predominantly from the P2 promoter (Fig. 1A, lane 3). This pattern of transcription initiation is typical of c-*myc* transcription in mammalian cells (Fig. 1A, lane 2) and in previous *Xenopus* oocyte assays (3, 54). The deletion construct, ΔP1, directed transcription only from the P2 promoter (Fig. 1A, lane 4); the deletion construct, ΔP2, resulted in transcription only from the P1 promoter (Fig. 1A, lane 5).

Mapping of 3′ ends of P1 and P2 transcripts from the same RNA samples was performed with end-labelled S1 probes (Fig. 1B). By using the Nci probe, which is end labelled at the *Nci*I site 66 bp downstream of P2 and, therefore, homologous to both P1 and P2 transcripts, a number of 3′ ends were mapped throughout the 3′ end of exon 1 and the 5′ end of intron 1 (Fig. 1B, lanes 3 to 5). These include the prominent sites, T1 and T2, described in previous studies (3). T1 and T2 are thymidine-rich sequences located on either side of the exon 1-intron 1 boundary. The other 3′ ends mapped with the Nci probe are indicated by asterisks in Fig. 1B. They do not map to thymidine-rich regions and lie within the 3′ half of exon 1, from +176 to +356 relative to P2. No prematurely terminated RNAs with 3′ ends in the first half of exon 1 were detected by using the Nci probe and shorter gel runs (data not shown). The band marked by × in the Nci probe samples (Fig. 1B, lanes 2 to 5) appears to be an artifact of this probe preparation, prominent when large amounts of total RNA were assayed (Fig. 1B, lanes 4 and 5). We have not observed a significant band at this position in other assays using this or similar RNAs and other probes (Fig. 1B, lane 8, and 2B, lane 3). The band labelled "spliced" results from a discontinuity between the probe and spliced c-*myc* RNA. This band and the band (FL) obtained with the fully protected probe together represent full-length c-*myc* RNAs synthesized during the transcription reaction. These data confirm the Northern blot analysis results reported previously, i.e., transcripts originating from the P1 promoter (ΔP2) are predominantly full length whereas transcripts originating from the P2 promoter (ΔP1) are a mixture of full length and terminated (54).

In order to map the 3′ ends of P1 transcripts alone from the WT RNA sample (which contains both P1 and P2 transcripts), an S1 probe, end labelled at the *Xho*I site between

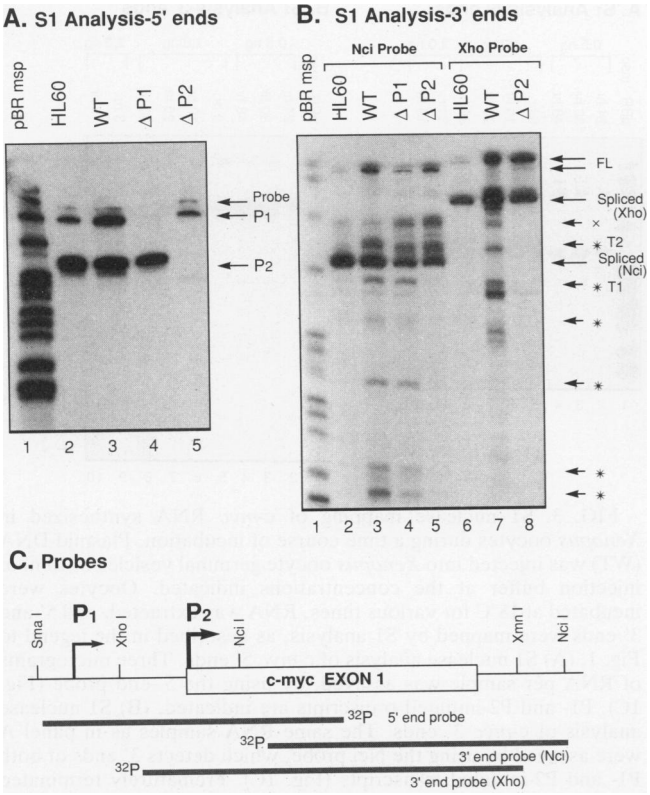

FIG. 1. S1 nuclease mapping of c-*myc* RNA synthesized in *Xenopus* oocytes. Approximately 1 ng each of plasmid DNA of three c-*myc* constructs was injected into *Xenopus* oocyte germinal vesicles, oocytes were incubated at 18°C for 24 h, and RNA was extracted. S1 analysis was performed on 3 μg of oocyte RNA per sample as described in Materials and Methods, using single-stranded, end-labelled probes. Total RNA was extracted from HL60 cells, and 7 μg was assayed. No protected fragments resulted from an S1 analysis of RNA from *Xenopus* oocytes injected with buffer only. (A) S1 nuclease analysis of c-*myc* 5′ ends. Transcripts initiating at the P1 promoter protect a 401-base fragment; transcripts initiating at the P2 promoter protect a 270-base fragment. The human c-*myc* plasmids WT, ΔP1, and ΔP2 are described in Materials and Methods and elsewhere in the text. (B) S1 nuclease analysis of c-*myc* 3′ ends. The Nci probe is end labelled at the *Nci*I site, 66 bp downstream of the P2 promoter and, therefore, detects both P1- and P2-initiated transcripts. The Xho probe is end labelled at the *Xho*I site between the P1 and P2 promoters and, therefore, detects only P1-initiated transcripts. The bands representing spliced c-*myc* RNA and full-length (FL), unspliced c-*myc* RNA are indicated. *, prematurely terminated c-*myc* RNAs, obtained with the Nci probe; T1 and T2, sites of premature termination described in previous studies (3); pBRmsp, pBR322 cut with *Msp*I. The band marked by × in the Nci probe samples (lanes 2 to 5) appears to be an artifact of this probe preparation. This band does not appear in S1 analyses using other Nci probe preparations with the same RNA samples. (C) Probes used in S1 analyses shown in Fig. 1 to 3 and 5. Probes are single-stranded, end-labelled DNA fragments prepared as described in Materials and Methods.

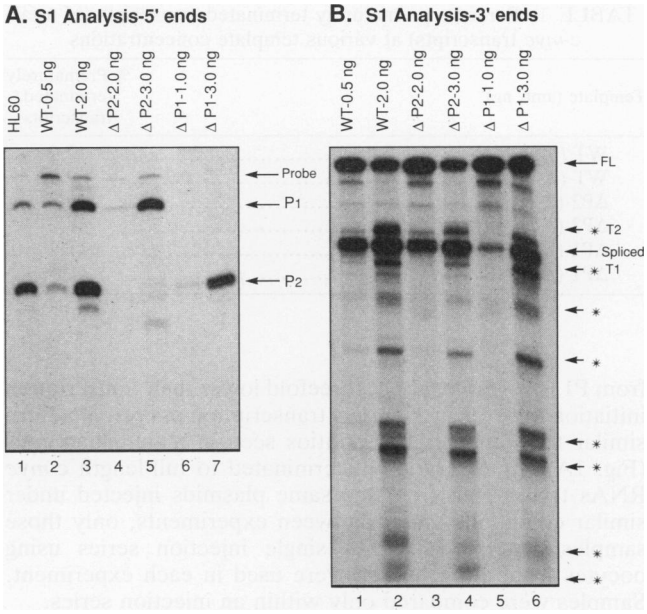

FIG. 2. S1 nuclease mapping of c-*myc* RNA synthesized in *Xenopus* oocytes after injection of low or high concentrations of c-*myc* DNA templates. Plasmid DNAs (WT, ΔP2, and ΔP1) were injected into *Xenopus* oocyte germinal vesicles at the concentrations indicated in 10 nl of injection buffer. Oocytes were incubated at 18°C for 24 h, RNA was extracted, and 5′ and 3′ ends were mapped by S1 analysis, as described in the legend to Fig. 1. (A) S1 nuclease analysis of c-*myc* 5′ ends. Three micrograms of RNA per sample was assayed by using the 5′-end probe (Fig. 1C). P1- and P2-initiated transcripts are indicated. (B) S1 nuclease analysis of c-*myc* 3′ ends. The same RNA samples as in panel A were assayed by using the Nci probe, which detects 3′ ends of both P1- and P2-initiated transcripts (Fig. 1C). Prematurely terminated RNAs (*) and spliced c-*myc* RNA are indicated. FL, full-length probe protected by unspliced c-*myc* RNA; T1 and T2, sites of premature termination described in previous studies (3). Amounts of RNA per sample were adjusted to approximately equalize the amount of spliced c-*myc* RNA and thereby facilitate visual comparison and quantitation of bands. Amounts of RNA (in micrograms) assayed per sample were as follows: lane 1, 12; lane 2, 4.5; lane 3, 30; lane 4, 4.5; lane 5, 30; lane 6, 12.

P1 and P2, was used (Fig. 1B, lanes 6 to 8). To our surprise, the sites and intensities of terminated and full-length RNAs that originated from the P1 promoter (Fig. 1B, lane 7) were the same as those of RNAs that originated from both promoters combined (Fig. 1B, lane 3). The end label of the Xho probe is 161 bases upstream of the end label of the Nci probe; therefore, the positions of the spliced RNA band and prematurely terminated RNA bands are shifted on the gel by 161 bp relative to those in the Nci probe lanes (Fig. 1B, lanes 3 to 5). The data for the Xho probe apparently contradict data from the 3′-end mapping of deletion constructs ΔP1 and ΔP2 obtained by using the Nci probe (Fig. 1B, lanes 4, 5, and 8) and from Northern analyses from the previous study (54), which indicated that P1 transcripts (transcribed from the ΔP2 plasmid) were primarily full length.

In summary, in the absence of the P2 promoter P1-initiated transcripts are predominantly full length, but in the presence of the P2 promoter P1-initiated transcripts show the same pattern of terminated and full-length RNAs as that seen for P2-initiated transcripts.

Effects of template concentration on c-*myc* premature termination. The different intensities of premature termination from the P1 promoter in the presence and absence of the stronger P2 promoter, as well as variability in the ratio of terminated to full-length RNAs from assay to assay (data not shown), led us to investigate the effects of transcription levels per se from either promoter on the efficiency of premature transcription termination. Transcription initiation

TABLE 1. Percents prematurely terminated transcripts (of total c-*myc* transcripts) at various template concentrations

Template (amt, ng)	% Prematurely terminated transcripts
WT (0.5)	17.9
WT (2.0)	49.9
ΔP2 (2.0)	15.8
ΔP2 (3.0)	49.0
ΔP1 (1.0)	11.5
ΔP1 (3.0)	48.5

from P1 is generally about threefold lower than transcription initiation from P2 in *Xenopus* transcription assays, a pattern similar to c-*myc* promoter ratios seen in mammalian cells (Fig. 1A). As the ratios of terminated to full-length c-*myc* RNAs transcribed from the same plasmids injected under similar conditions varied between experiments, only those samples generated from a single injection series using oocytes from a single frog were used in each experiment. Samples were compared only within an injection series.

Various concentrations of c-*myc* plasmids WT, ΔP1, and ΔP2 were injected into *Xenopus* oocyte germinal vesicles, oocytes were incubated for 24 h, and RNA was extracted. RNA start sites and 3′ ends were determined by S1 analysis as described above.

Results of 5′-end mapping are shown in Fig. 2A. The same amount of RNA (3 μg) was assayed in each lane. The c-*myc* gene was accurately and efficiently transcribed from the P1 and P2 promoters in *Xenopus* oocytes, and transcription was entirely due to RNA polymerase II, as determined by α-amanitin controls in parallel nuclear runoff transcription assays on isolated germinal vesicles (data not shown). An injection concentration yielding high and low levels of c-*myc* transcript for each c-*myc* construct was selected for further study.

Mapping of the 3′ ends of these RNA samples is shown in Fig. 2B. Amounts of RNA assayed per sample were adjusted to approximately equalize the spliced RNA signal, thereby facilitating visual comparison. Prematurely terminated c-*myc* RNAs are indicated with an asterisk. Figure 2B shows that the ratio of prematurely terminated to full-length RNAs is low when small amounts of c-*myc* DNA are injected into *Xenopus* oocytes and is high when larger amounts of DNA are injected. These results apply to transcription originating from either the P1, the P2, or both promoters together.

In order to quantitate these results, bands were excised from the S1 gel shown in Fig. 2B following autoradiography and counted in a scintillation counter. Table 1 shows percents of prematurely terminated RNA species (of total c-*myc* RNA). When small amounts of c-*myc* plasmids were injected into *Xenopus* oocytes, less than 18% of c-*myc* transcripts from either the P1 or the P2 promoter prematurely terminated. However, when larger amounts of c-*myc* plasmids were injected into *Xenopus* oocytes, approximately 50% of total c-*myc* transcripts prematurely terminated.

These data show that the ratio of prematurely terminated to full-length c-*myc* RNAs from either the P1 or the P2 promoter correlates with the amount of template injected.

Effects of incubation time on premature transcription termination. To investigate whether the amount of premature termination was related to the amount of transcription or simply to the quantity of DNA injected, we performed a transcription time course experiment. WT c-*myc* plasmid

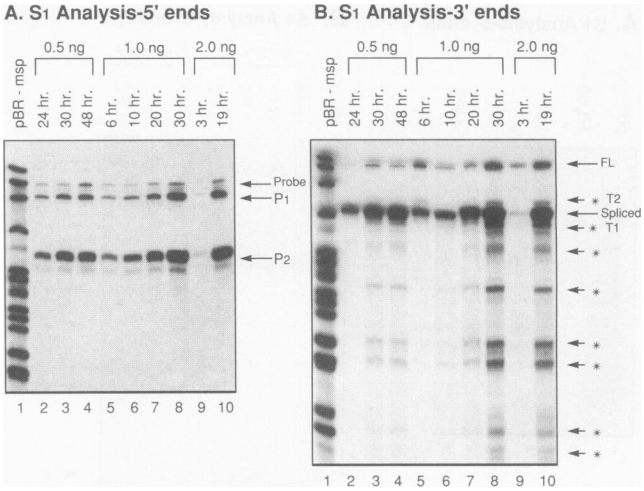

FIG. 3. S1 nuclease mapping of c-*myc* RNA synthesized in *Xenopus* oocytes during a time course of incubation. Plasmid DNA (WT) was injected into *Xenopus* oocyte germinal vesicles in 10 nl of injection buffer at the concentrations indicated. Oocytes were incubated at 18°C for various times, RNA was extracted, and 5′ and 3′ ends were mapped by S1 analysis, as described in the legend to Fig. 1. (A) S1 nuclease analysis of c-*myc* 5′ ends. Three micrograms of RNA per sample was assayed, by using the 5′-end probe (Fig. 1C). P1- and P2-initiated transcripts are indicated. (B) S1 nuclease analysis of c-*myc* 3′ ends. The same RNA samples as in panel A were assayed by using the Nci probe, which detects 3′ ends of both P1- and P2-initiated transcripts (Fig. 1C). Prematurely terminated RNAs (*) and spliced c-*myc* RNA are indicated. FL, full-length probe protected by unspliced c-*myc* RNA. Three micrograms of RNA per sample was assayed.

was injected into *Xenopus* oocyte germinal vesicles at three different concentrations, and oocytes were incubated for the times indicated in Fig. 3. RNA was extracted and assayed by S1 analysis as described above.

Figure 3A shows 5′ ends of c-*myc* transcripts synthesized in this experiment. The same amount (3 μg) of oocyte RNA per sample was assayed. A transcription standard was not coinjected in these experiments to eliminate the complication of introducing competing transcription from another promoter, as described below. Figure 3A shows that c-*myc* transcripts accumulated at different rates for each of the amounts injected over a 30-h incubation time. Accumulation of transcripts ceased between 30 and 48 h of incubation (Fig. 3A, lanes 3 and 4).

Figure 3B shows a 3′-end mapping of the RNA samples shown in Fig. 3A. Bands were excised and counted in a scintillation counter, and counts per minute of full-length, spliced, and prematurely terminated RNAs were determined during the time course. Percents prematurely terminated transcripts (of total c-*myc* RNA transcripts) are shown in Table 2.

Data in Fig. 3B and Table 2 demonstrate that the ratio of prematurely terminated to full-length c-*myc* RNAs increased as the total amount of transcript accumulated during the time of incubation for all three template concentrations injected. However, as with the 0.5-ng injections (Fig. 3B, lanes 2 to 4), the time of incubation alone was not sufficient to result in high levels (>40%) of premature termination. Rather, a critical threshold of transcript accumulation (e.g., 1 ng for 30 h or 2 ng for 19 h) appeared to be necessary to obtain more than 40% prematurely terminated c-*myc* RNAs.

TABLE 2. Accumulation of prematurely terminated c-*myc* transcripts over the time course of incubation

WT template amt (ng)	% Prematurely terminated transcripts[a] after the following incubation time (h):							
	3	6	10	19	20	24	30	48
0.5						0[b]	40.3	37.8
1.0		23.9	26.4		38.1		56.2	
2.0	0[b]			55.0				

[a] Of total c-*myc* transcripts. Values were calculated from counts per minute of gel slices.
[b] Undetectable.

Nuclear runoff transcription assays of time course transcription. In order to determine whether modulation in premature transcription termination was a transcriptional rather than a posttranscriptional effect, we performed nuclear runoff transcription assays on isolated germinal vesicles from oocytes injected in a time course experiment. Products of the runoff assay were hybridized to blots containing single-stranded probes covering the human c-*myc* upstream, exon 1, and intron 1 regions. Figure 4 shows the results of the nuclear runoff assays. Transcription signals were eliminated by inclusion of 2 μg of α-amanitin per ml, indicating transcription by RNA polymerase II (data not shown). The pBR322 and H2b plasmids were included as positive controls for RNA polymerase II transcription in these injections. Transcription arising from vector sequences has been observed previously in runoff transcription assays of nuclei from injected *Xenopus* oocytes (54), presumably because of transcription initiating from cryptic promoters within the plasmids. Similar positive transcription signals to vector sequences have been observed with runoff transcription assays of nuclei from cell lines stably transfected with c-*myc* plasmids (54). The greater intensity of the c-*myc* exon 1 signal in all samples in Fig. 4, compared with that of the 5′ upstream signal, indicates that the majority of exon 1 and intron 1 transcription was initiated from the c-*myc* P1 and P2 promoters rather than from the upstream minor promoter, P0 (2), or from vector sequences. The exon 1 and intron 1 signals were quantitated by densitometry of autoradiographs and compared after adjusting the Ex 1 signal 1.86-fold to correct for the lower uridine content of sense transcripts within the Ex 1 probe region.

After a 21-h incubation, 0.5 ng of injected template directed read-through transcription (Fig. 4A). The Ex 1-to-Int 1 signal ratio was 0.76:1 by densitometry with correction for uridine content, indicating that RNA polymerases were present to similar degrees in exon 1 and intron 1. This result is consistent with the S1 analysis of steady-state RNA in Fig. 3, which shows predominantly full-length c-*myc* RNA in the 0.5-ng–24-h sample (Fig. 3B, lane 2) and low levels of c-*myc* steady-state transcripts (Fig. 3A, lane 2).

After an 18-h incubation (Fig. 4B), 1.0 ng of injected template directed a moderate transcription block. The Ex 1-to-Int 1 signal ratio was 4.4:1 by densitometry with correction for uridine content. Consistent with this result, the steady-state RNA after a 20-h incubation contained approximately 38% terminated c-*myc* RNAs (Fig. 3B, lane 7, and Table 2).

After an 8-h incubation, 2 ng of injected template directed read-through transcription (Fig. 4C). The Ex 1-to-Int 1 signal ratio was 1.2:1 by densitometry with correction for uridine content. This is consistent with the presence of low levels of

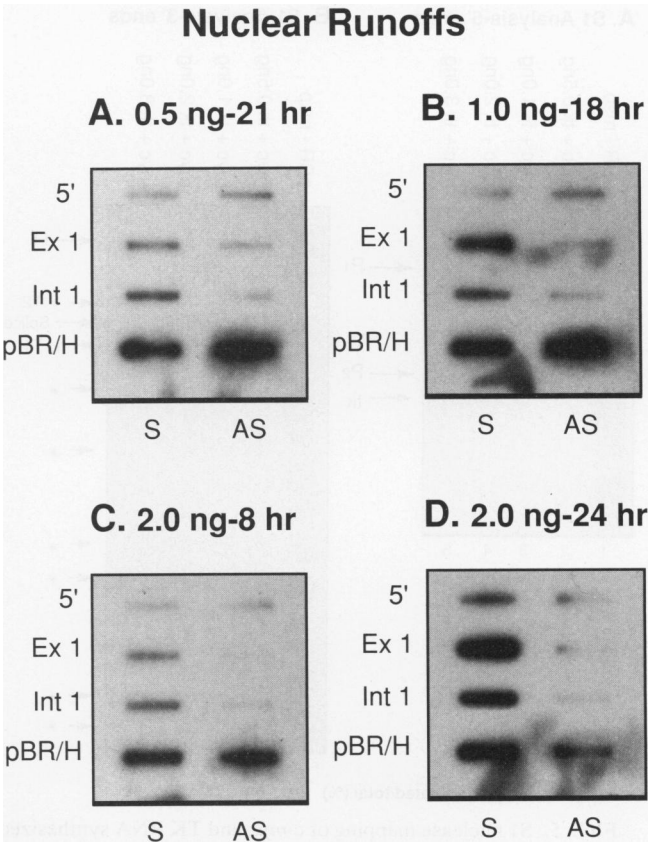

FIG. 4. Nuclear runoff transcription assays of *Xenopus* oocyte germinal vesicles dissected during a time course. Plasmid DNA (WT) was injected into *Xenopus* oocyte germinal vesicles in 10 nl of injection buffer at the concentrations indicated. Oocytes were incubated at 18°C for the times indicated, and germinal vesicles were manually dissected as described in Materials and Methods. Products of the nuclear runoff transcription assays were hybridized to immobilized single-stranded DNA probes which detect sense (S) and antisense (AS) transcripts from the following regions: 5′, a 568-base fragment upstream of the P1 promoter; Ex 1, a 445-base fragment within c-*myc* exon 1; and Int 1, a 606-base fragment within c-*myc* intron 1. Uridine contents of sense RNAs in these probe regions are 121, 73, and 136 bases, respectively. Double-stranded plasmids pBR322 (pBR; lanes S) and histone H2b (H; lanes AS) were included as indicators of RNA polymerase II transcription level in each assay.

prematurely terminated c-*myc* RNAs in the 2-ng–3-h sample (Fig. 3B, lane 9, and Table 2). In contrast, after a 24-h incubation, 2 ng of injected template directed a strong transcription block (Fig. 4D). The Ex 1-to-Int 1 signal ratio was 4.5:1 by densitometry with correction for uridine content. In keeping with this result, 55% prematurely terminated c-*myc* RNAs was measured for the 2-ng–19-h sample (Fig. 3B, lane 10, and Table 2).

In summary, the nuclear runoff transcription data reflect the accumulation of steady-state RNA in the time course experiments. These data, coupled with the steady-state S1 analyses from the time course and template concentration experiments, strongly suggest that the level of premature termination in the *Xenopus* oocyte assay is a transcriptional effect and is not due to degradation, processing, or a titration effect resulting directly from high levels of injected template.

Coinjection of TK plasmid. In order to determine whether

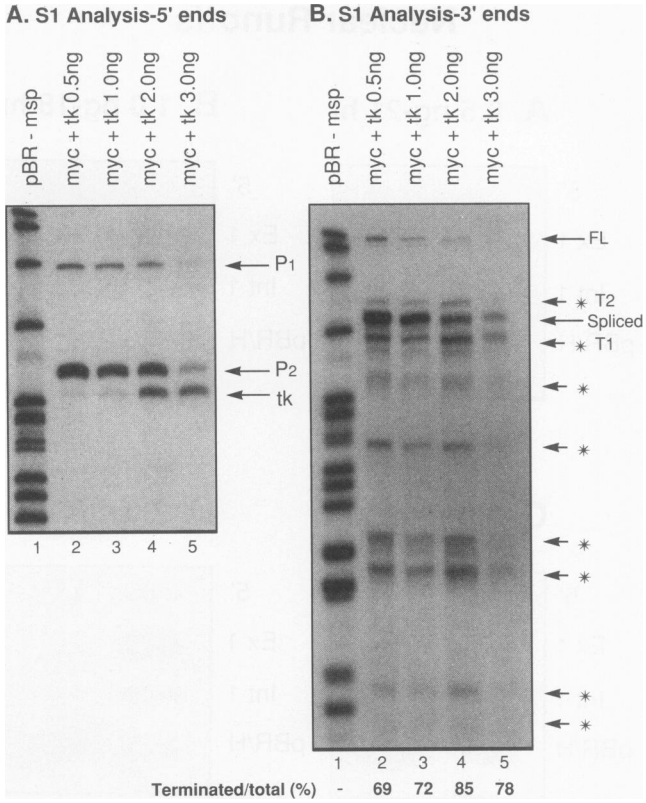

FIG. 5. S1 nuclease mapping of c-*myc* and TK RNA synthesized after coinjection of c-*myc* and TK plasmids into *Xenopus* oocyte germinal vesicles. WT c-*myc* plasmid (1 ng per sample) was coinjected with the herpes simplex virus TK plasmid at the concentrations indicated into *Xenopus* oocyte germinal vesicles in a total volume of 10 nl per oocyte. Oocytes were incubated at 18°C for 24 h and RNA was extracted and assayed by S1 analysis as described in the legend to Fig. 1. (A) S1 nuclease analysis of c-*myc* and TK 5′ ends. Three micrograms of oocyte RNA per sample was assayed using the 5′-end probe (Fig. 1C) and the end-labelled TK probe, as described in Materials and Methods. Transcripts initiating at the c-*myc* P1 and P2 promoters and at the TK promoter are indicated. The c-*myc* and TK probes were of different specific activities. (B) S1 nuclease analysis of c-*myc* 3′ ends. Three micrograms each of the same oocyte RNAs as in panel A was mapped by using the Nci probe, which detects 3′ ends of c-*myc* transcripts initiating at both P1 and P2 promoters (Fig. 1C). Prematurely terminated RNAs (*) and spliced c-*myc* RNA are indicated. FL, full-length probe, protected by unspliced c-*myc* RNA; T1 and T2, sites of premature termination described in previous studies (3). Bands were excised and counted in a scintillation counter. The percents prematurely terminated transcripts (of total c-*myc* RNAs) are indicated at the bottom.

the effects upon transcription elongation were specific to the c-*myc* promoter or were due to a more general effect related to transcription per se, we performed coinjection experiments.

WT c-*myc* plasmid (1 ng) was coinjected with increasing amounts of TK plasmid (herpes simplex virus TK gene, including the TK promoter and 585 bp of transcribed sequence, cloned into pVZ1) into *Xenopus* oocyte germinal vesicles, oocytes were incubated for 24 h, and RNA was extracted and assayed by S1 analysis, as described above. The TK promoter is accurately and efficiently transcribed in *Xenopus* oocytes by RNA polymerase II (3, 16). Figure 5A shows 5′-end mapping of c-*myc* and TK transcripts from coinjected oocytes. The same amount (3 μg) of oocyte RNA per lane was assayed, and bands were excised and counted in a scintillation counter. As the amount of injected TK plasmid increased (up to 2 ng), TK transcription levels increased and c-*myc* transcription levels decreased, suggesting competition between promoters for limited transcription components. Above a total of 3 ng of injected DNA, both c-*myc* and TK transcription levels decreased. This repressing effect of high DNA concentrations on transcription in *Xenopus* oocytes has been described previously (34, 55) and may result from a titration and partitioning of limited transcription initiation factors between available DNA templates. Figure 5B shows 3′-end mapping of c-*myc* RNAs from the same samples as in Fig. 5A. The same amount (3 μg) of oocyte RNA per sample was assayed. Bands were excised and counted in a scintillation counter. Percents prematurely terminated transcripts (of total c-*myc* transcripts) were determined and are indicated in Fig. 5B.

Data in Fig. 5B show that the percent prematurely terminated c-*myc* transcripts increased and remained high as transcription from the TK promoter increased, even though the amount of c-*myc* template DNA remained constant and c-*myc* transcription levels decreased.

These results demonstrate that the effects of transcription initiation levels on premature transcription termination are not restricted to transcription from the c-*myc* promoter. Premature transcription termination within the c-*myc* gene can be enhanced through competing transcription from a heterologous promoter.

DISCUSSION

In this study, we have shown that the extent of premature transcription termination from the human c-*myc* gene in *Xenopus* oocytes is dependent upon transcription initiation levels. When transcription levels are low, because of low amounts of DNA template injected or short incubation times, low levels of premature transcription termination occur. Conversely, when the amount of transcription increases, because of injection of larger amounts of DNA template, increased incubation times, or coinjection with a heterologous promoter, the ratio of prematurely terminated to full-length transcripts increases.

These data are consistent with the hypothesis that one or more general RNA polymerase II transcription elongation factors are limiting in *Xenopus* oocytes and may be more limiting than are RNA polymerase II transcription initiation factors.

The limiting factor appears not to be ATP or another ribonucleotide, as coinjection of 1 mM ribonucleoside triphosphates does not relieve premature transcription termination at 1 or 2 ng of c-*myc* DNA injected (data not shown).

The limiting transcription elongation factor(s) may be one of the known general RNA polymerase II elongation factors, such as TFIIS or TFIIX (1, 6, 42, 51). The transcription elongation factor, TFIIF, is less likely as a candidate for the oocyte limiting factor, as it is also an essential transcription initiation factor (4, 5, 39). The limiting elongation factor(s) may operate on the transcription apparatus prior to or after initiation but is not essential for transcription initiation itself. The elongation function appears to be intimately involved with the transcription process itself, as its depletion requires active transcription, not simply the presence of potentially active promoters, as seen at early incubation times in the

time course experiment (Fig. 3). A *Xenopus* homolog of the recently identified factor, P-TEF, which acts early after transcription initiation, is sensitive to 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and is limiting in *Drosophila* extracts (31), may also be a limiting elongation factor in *Xenopus* oocytes. A recent study has demonstrated that premature transcription termination from the c-*myc* promoters is greatly enhanced in *Xenopus* oocytes in the presence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (45). Reconstitution of *Xenopus* oocytes with purified or fractionated transcription elongation factors may provide clues to the identity of the limiting elongation factor.

The conclusions from our study are consistent with those of Middleton and Morgan (34), who observed premature transcription termination from the *X. laevis* α-tubulin gene after injection into *Xenopus* oocyte germinal vesicles. At low template concentrations or at early incubation times, little premature termination from the α-tubulin template occurred. However, as the amount of template increased or the incubation time increased, the proportion of terminated to full-length RNAs also increased. The authors hypothesized an antitermination activity that binds to promoters and is necessary during initiation to assemble an elongation-competent transcription complex. Our results extend their findings and demonstrate that this limitation of antitermination or elongation functions is more general than previously thought, also operating on RNA polymerase II promoters from human and virus genes transcribed in the *Xenopus* system.

The results from the present study have implications for several investigations dealing with transcription elongation. Firstly, in previous studies, we used the *Xenopus* oocyte injection assay to characterize the block to transcription elongation that occurs in the first exon and intron of the human c-*myc* gene (54). These studies suggested that after injection into *Xenopus* oocytes transcription from the c-*myc* P1 promoter resulted in read-through (full-length) transcripts whereas transcription from the stronger P2 promoter resulted in a combination of prematurely terminated and full-length transcripts. The data in the present study demonstrate that promoter-specific processivity in this assay system most likely resulted from differences between the overall amounts of transcription occurring from the two promoters when transcribed separately from either the ΔP1 or the ΔP2 plasmid. When the weaker P1 promoter is transcribed in the presence of the stronger P2 promoter or when transcription levels are high, transcription from the P1 promoter leads to high levels of prematurely terminated transcripts, similar to P2-initiated transcription. In support of our present data, Wright et al. (59) previously observed that transcripts initiating from the P1 promoter of the mouse c-*myc* gene were capable of efficient premature termination when expressed in the *Xenopus* oocyte transcription system.

We would like to point out that these conclusions do not alter the hypothesis that promoter-specific effects may operate on the c-*myc* transcription elongation in mammalian cells. Indeed, Burkitt's lymphoma and HeLa cells consistently show a switch in promoter usage to P1 and a loss of the c-*myc* transcription block (52, 54). Also, a recent study (33) has demonstrated a link between the presence of sequence elements downstream of the P1 promoter and the transcription elongation properties of P1-initiated transcription. Similar but independently regulated elements downstream of the P2 promoter may operate to modulate P2 transcription elongation. In addition, reports on the c-*myc* block to transcription elongation in the mouse c-*myc* gene show a role for the ME1a1 element in the P2 promoter in controlling the P2 block to transcription elongation (15, 35). Therefore, the relationship between events at a promoter and downstream transcription elongation may be important in transcriptional regulation of the c-*myc* gene. Promoter-specific effects on transcription termination and elongation are well documented in the small nuclear RNA genes U1 and U2 (18, 19, 38) and in transcription extracts (58).

Secondly, the *Xenopus* oocyte system has been used to study transcription elongation in a number of genes, including the human c-*myc* gene (3, 54), mouse c-*myc* gene (3, 45, 59), human and mouse adenosine deaminase genes (8, 9, 40), and α-tubulin gene (34). The present study provides a word of caution about the use of the *Xenopus* transcription system in studies of transcription elongation. Premature transcription termination may be absent or high, depending upon the amount of transcription occurring in the assay, which may, in turn, depend upon the amount of template injected, incubation time, and promoter strength. Coinjection of standards may also alter the ratios of prematurely terminated to full-length transcripts. Effects of transcription initiation levels on transcription elongation may account for several previous observations of c-*myc* transcription patterns in the *Xenopus* oocyte system. However, interpretation of results from most previous *Xenopus* transcription assays is complicated by the fact that overall transcription levels were not assessed or controlled in these studies. Therefore, the extent to which transcription initiation levels contributed to the efficiency of premature termination in previous studies is difficult to assess. The *Xenopus* transcription system is useful for identifying sites of intrinsic pausing or termination by RNA polymerase II, and many of the terminated RNAs from the first exon of c-*myc* correspond to intrinsic-termination sites observed in in vitro systems (24). However, it must be determined whether these termination sites operate in vivo under conditions in which transcription elongation factors may not be limiting. Also, the presence of limiting transcription elongation factors may become an important consideration in the interpretation of in vitro transcription studies, as under some conditions (template excess, incubation time, or promoter strength) transcription elongation factors may become limiting before transcription initiation factors. Marshall and Price (31) have observed such in vitro transcription conditions, in which the *Drosophila* transcription factor, P-TEF, is limiting. In addition, the adenovirus major late promoter premature termination site, T+, is inefficiently recognized when placed downstream of the β-globin promoter in vitro (58), but termination can be enhanced by titration with excess β-globin template (17).

Finally, the finding that one or more transcription elongation functions are limiting in *Xenopus* oocytes suggests a mechanism whereby the availability or activity of a transcription elongation factor could contribute to regulated gene expression in other eukaryotic cells. As mentioned above, transcription elongation factors are limiting in both *Drosophila* and human transcription extracts and may, under certain conditions, become limiting in vivo. Global alterations in transcription occur during phases of the cell cycle (11, 49), and several essential genes involved in growth control (e.g., c-*myc*, c-*fos*, and c-*myb*) are known to be regulated by transcription elongation. Therefore, it would be of considerable interest to determine whether transcription efficiency from a set or subset of promoters could be controlled by limiting or modified transcription elongation factors in these regulatory situations.

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