Promoter-proximal pausing of RNA polymerase II defines a general rate-limiting step after transcription initiation

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We have shown previously that the majority of RNA polymerase II complexes initiated at the *c-myc* gene are paused in the promoter-proximal region, similar to observations in the *Drosophila hsp70* gene. Our analyses define the TATA box or initiator sequences in the *c-myc* gene as necessary components for the establishment of paused RNA polymerase II. Deletion of upstream sequences or even the TATA box does not influence significantly the degree of transcriptional initiation or pausing. Deletion of both the TATA box and sequences at the transcription initiation site, however, abolishes transcriptional pausing of transcription complexes but still allows synthesis of full-length RNA. Further analyses with synthetic promoter constructs reveal that the simple combination of upstream activator with TATA consensus sequences or initiator sequences act synergistically to recruit high levels of RNA polymerase II complexes. Only a minor fraction of these complexes escapes into regions further downstream. Several different *trans*-activation domains fused to GAL4–DNA-binding domains, including strong activators such as VP16, do not eliminate promoter-proximal pausing of RNA polymerase. Thus, we conclude that pausing of RNA polymerase II is a common phenomenon in eukaryotic transcription and does not require complex promoter structures. Further analyses reveal that enhancers have a modest influence on transcription initiation and on release of transcription complexes out of the pause site but may function primarily to increase the elongation competence of transcription complexes.

[Key Words: *C-myc* gene; promoter-proximal pausing; RNA polymerase II; transcription initiation; elongation; enhancer]

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The level of *c-myc* RNA is regulated largely through a mechanism that operates after transcription initiation. This conclusion is based on the observation that steady-state *c-myc* RNA levels decline significantly as cells differentiate, whereas transcriptional initiation, as measured in nuclear run-on assays, remains unchanged (Bentley and Groudine 1986; Eick and Bornkamm 1986; Nepveu and Marcu 1986; for review, see Spencer and Groudine 1990; Krumm et al. 1993).

Recent *in vivo* assays have demonstrated paused RNA polymerase II complexes at promoter-proximal regions of the *c-myc* gene. For example, *in vivo* footprinting with KMnO₄ as a reagent to detect single-stranded DNA residues within transcription bubbles revealed hyper-reactive sites around position +30. These results suggested that RNA polymerases pause in the promoter-proximal region around +30. Nuclear run-on experiments with short probes confirmed that the majority of RNA polymerases is located within the first 169 bp (Krumm et al. 1992). In another study, nuclear run-on experiments with oligonucleotide probes as short as 50 bp also indi-

cated that the highest level of RNA polymerase II is within the promoter-proximal region between +1 and +50 (Strobl and Eick 1992). Further experiments demonstrated that sequences 5' of +47 were sufficient to confer promoter-proximal pausing (Krumm et al. 1992).

Promoter-proximal pausing of RNA polymerase II has also been observed in the *hsp70* and other genes in *Drosophila* (Rougvie and Lis 1988; O'Brien and Lis 1991; for review, see Lis and Wu 1993) and may be responsible for polarity of transcription observed in other genes such as the human immunodeficiency virus–long terminal repeat (HIV–LTR) (Kao et al. 1987; Laspias et al. 1989; for review, see Cullen 1993). Similar to the *c-myc* gene, transcription initiation in the *Drosophila hsp70* gene appears to be unchanged in induced (heat-shocked) and noninduced (nonheat-shocked) cells. In both *c-myc* and *hsp70*, promoter-proximal pausing is detected in nuclear run-on assays by the high salt- or Sarkosyl-mediated release of RNA polymerase complexes paused at or immediately downstream of the transcription initiation site. Results of nuclear run-on assays with the *hsp70* gene correlate with those of UV cross-linking assays where high amounts of RNA polymerase II were cross-linked to

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promoter-proximal DNA, and only little was detected on downstream sequences (Rougvie and Lis 1988; O'Brien and Lis 1991). In addition, *in vivo* footprinting with KMnO_4 and sizing of short RNAs generated in nuclear run-ons further confirmed that RNA polymerase II pauses at promoter-proximal regions (Giardina et al. 1992; Rasmussen and Lis 1993).

Pausing of RNA polymerase II has been found to be a target for regulation of gene expression in prokaryotes. For example, promoter-proximal pausing is an important component of the antitermination mechanism at the λ pR' promoter, where RNA polymerase pauses 16 bases downstream of the initiation site and requires the Q protein to escape the pause site and resume elongation (for review, see Greenblatt et al. 1993; Roberts 1993). The interaction of the transcription complex with the Q protein also results in the suppression of termination signals farther downstream. In addition, the antitermination activity of the λ N protein has been suggested to rely on the suppression of pausing of polymerase at the Rho-dependent λ terminator (Mason et al. 1992).

Here, we present experiments that delineate the sequences involved in promoter-proximal pausing of RNA polymerase II complexes in mammalian cells. In reconstitution experiments, we show that promoter-proximal pausing occurs to a high level in simple promoter constructs. Both core promoter sequences and upstream activators synergize to recruit high levels of transcription complexes pausing within promoter-proximal regions. The formation of paused RNA polymerase II complexes is supported by different classes of upstream activators. This indicates that pausing of transcription complexes is very common and a major rate-limiting mechanism in gene expression. We also show that enhancers and some upstream activators have only a modest effect on transcription initiation and on the release of transcription complexes out of the promoter-proximal pause site; these elements may increase the amount of full-length RNA through modulation of elongation competence of RNA polymerase II, as suggested previously (Krumm et al. 1993; Madisen and Groudine 1994; Yankulov et al. 1994).

Results

Promoter-proximal pausing functions in the absence of the c-myc consensus TATA box

Promoter-proximal pausing of RNA polymerase II in the *c-myc* gene is also observed when a human *c-myc* construct is transfected into mouse L cells (Krumm et al. 1992). In these experiments, high amounts of transcription complexes were detected in promoter-proximal regions, and very few RNA polymerase II complexes were found farther downstream. The experimental strategy to identify sequence elements responsible for the generation of paused RNA polymerase included construction of linker scanner and deletion mutants within the human *c-myc* P2 promoter. After transfection of these constructs into mouse L cells, polyclonal lines were estab-

lished and the extent of promoter-proximal pausing was measured in nuclear run-on assays. In this assay, transcriptionally engaged RNA polymerase II complexes in isolated nuclei continue to elongate nascent RNA for an average of 100 to 200 nucleotides. Similar to observations at the *Drosophila hsp70* gene (Rougvie and Lis 1988), RNA polymerase II complexes paused in promoter-proximal regions in the *c-myc* gene are released and resume elongation only in the presence of a salt concentration of 150 mM or Sarkosyl (Krumm et al. 1992). The resultant labeled RNA is hybridized to "cold" probes spanning different regions of the gene. Because no reinitiation occurs under the nuclear run-on conditions used in these experiments, this assay measures the distribution of transcription complexes within a gene at the time of nuclear isolation.

The deletion of sequences upstream of the *c-myc* P2 promoter did not influence the degree of promoter-proximal pausing in comparison to the wild-type *c-myc* template (data not shown). Further analysis with mutant constructs revealed that sequences within the core promoter are involved in promoter-proximal pausing of transcription complexes. Single base mutations within the TATA box have been reported to decrease initiation in *Xenopus* oocyte injection experiments; concomitantly, the relative amount of RNA polymerases reading through termination/pause signals further downstream was increased (Meulia et al. 1993; Yankulov et al. 1994). The complete deletion of the TATA consensus sequence, however, abolished transcriptional activity in these assays (Spencer et al. 1990; Meulia et al. 1993). To test whether this holds true in mammalian cells, we transfected a full-length human *c-myc* construct containing a deletion of the TATA box of the P2 promoter (Spencer et al. 1990) into mouse L cells. Stably transfected, polyclonal cells containing similar numbers of integrated copies were analyzed in a nuclear run-on assay. Surprisingly, deletion of the TATA consensus sequence ($\Delta\text{P1}\Delta\text{P2TATA}$) did not abolish the recruitment of RNA polymerase II complexes. The promoter-proximal signal as detected by probe B (+47 to +169) in the nuclear run-on assay was reduced only twofold compared with the control construct ΔP1 (Fig. 1A). The nuclear run-on pattern of the $\Delta\text{P1}\Delta\text{P2TATA}$ construct is very similar to the pattern observed at the wild-type P2 promoter (ΔP1 ; Fig. 1A). Transcriptional activity is very high in promoter-proximal regions, as detected by probe B, whereas a very small amount of transcription complexes is detected farther downstream, with probes C (+170 to +349), D (+349 to +548), and E (+775 to +911). The high signal observed in the absence of a TATA box was sensitive to low α -amanitin (2 $\mu\text{g}/\text{ml}$) concentrations (data not shown). Thus, the promoter-proximal signal at the TATA-less *c-myc* promoter is attributable to RNA polymerase II. These results indicate that even in the absence of the TATA consensus sequence, the *c-myc* promoter can initiate transcription efficiently. These initiated transcription complexes, however, are as inefficient in elongation as at the wild-type P2 promoter. Thus, the *c-myc* TATA consensus se-

Figure 1. The TATA consensus sequence or sequences at the initiation site of the *c-myc* promoter are necessary for the formation of paused RNA polymerase. (A) Mouse fibroblasts (L cells), stably transfected with different *c-myc* constructs containing a wild-type P2 promoter ($\Delta P1$), a deletion of the TATA sequence ($\Delta P1\Delta P2TATA$), or the initiation region ($\Delta P1\Delta -11/+57$) or both the TATA box and the initiation site ($\Delta P1\Delta -48/+57$), were analyzed in nuclear run-on assays. Transfected cells were pooled and nuclei were isolated. Nuclear run-on assays were performed in the presence of radioactively labeled UTP, and nascent RNA was isolated and hybridized to single-stranded probes A–E to detect *c-myc* sense (+) and antisense (–) transcription. The structure of the *c-myc* gene and the position of probes are shown below. $\Delta P1$ refers to the deletion of a 30-bp sequence at the P1 promoter located upstream of the P2 promoter (Spencer et al. 1990). The presence of either a TATA box or the initiation region, or both, is required to generate a high level of transcription complexes paused in the promoter-proximal region (probe B). However, only little signal is detected with probes C, D, and E, indicating that only a small fraction of initiated transcription complexes transcribes regions further downstream. Probes detecting transcription of the actin or the H2B gene were used as internal controls. (B) Quantitation of nuclear run-on signals. The amount of radioactivity bound to each probe was determined using a PhosphorImager system, corrected for the uridine-content of each probe, and normalized to the signals obtained with the actin probe and to the number of integrated copies determined by Southern analyses (not shown). (C) Similarity of sequences at the *c-myc* initiation site to initiator sequences in the adenovirus major late promoter (AdMLP) and the terminal deoxynucleotidyl transferase gene (Tdt). The sites of transcription initiation are underlined. The *c-myc* Inr2 sequence is shown in an inverted orientation beginning at position +39 to +31 of the noncoding strand. (D) Nuclease S1 analysis of steady-state *c-myc* RNA of transfected mouse L cells. Total RNA isolated from transfected cell lines was analyzed in a S1 nuclease assay. End-labeled single-stranded probes were homologous to sequences of the 5' end of the human *c-myc* constructs. Probes homologous to the mouse GAPDH gene were included as an internal control. The diagram of transfected *c-myc* constructs containing deletions within the core promoter region is shown below. Solid arrows indicate the main sites of transcription initiation; open arrows indicate start sites detected after prolonged exposure of autoradiographs. (E2F) A binding site for the transcription activator E2F (Thalmeier et al. 1989). Single-stranded S1 probes extend from position +141 to the *Xho*I site at –95 of the *c-myc* wild-type sequence and were homologous for each construct except for $\Delta P1\Delta P2TATA$, where the same probe as for $\Delta P1$ was used. Arrows indicate probes protected by *c-myc* RNA; the star indicates protected transcripts originating from sites upstream of the P2 initiation site. Although the deletion of both the TATA and the initiator sequence ($\Delta P1\Delta -48/+57$) results in very little promoter-proximally paused RNA polymerase, full-length RNA can be detected in the absence of core promoter sequences. Further deletion of sequences up to –66 ($\Delta P1\Delta -66/+57$) results in the loss of detectable full length RNA.

quence is not required for promoter-proximal pausing, suggesting that other elements are responsible or can substitute for TATA in setting up paused RNA polymerase complexes.

The high efficiency of transcription complex recruitment even in the absence of a TATA box is reminiscent of other highly active eukaryotic promoters lacking TATA sequences. These promoters contain initiator se-

quences [Inr] that function efficiently to recruit and position RNA polymerase II complexes at the transcription initiation site (Smale and Baltimore 1989; for review, see Smale 1994). We noticed that the *c-myc* core promoter contains homologies to initiator sequences (Krumm et al. 1993). Similar to the HIV-LTR and the adenovirus major late promoter (Du et al. 1993), the *c-myc* transcription initiation region bears homologies to initiator sequences at two positions: sequences from -2 to $+8$ (Inr1) and $+31$ to $+39$ (Inr2). The Inr2 sequence is present in a reversed orientation relative to Inr1 (Fig. 1C). To determine whether Inr-like elements within the *c-myc* core promoter function to recruit transcription complexes that are inefficient in elongation, we deleted either one or both Inr-like elements of the P2 promoter. Neither the deletion of a single Inr-like element (data not shown) nor the deletion of the region between positions -11 to $+57$, encompassing both Inr-like elements, abolished promoter-proximal pausing (Fig. 1A). However, promoter-proximal pausing was not detectable in constructs where both the TATA consensus sequence and the Inr sequences (deletion from -48 to $+57$) were removed.

To correlate the level of transcriptional pausing with the production of full-length RNA, we performed S1 nuclease assays with probes derived from the 5' end of each construct (Figs. 1D and 2B). [Results were confirmed with probes derived from exon 2 (data not shown).] To our surprise, full-length RNA is synthesized by deletion construct $\Delta P1\Delta -48/+57$, although no promoter-proximally paused RNA polymerase is detectable in nuclear run-ons. This result demonstrates that the *c-myc* upstream sequences can direct the synthesis of full-length RNA in the absence of recognizable TATA or Inr sequences. Similar amounts of full-length RNA are detected in wild-type and deletion constructs encompassing TATA ($\Delta P1\Delta P2TATA$) and/or Inr sequences ($\Delta P1\Delta -11/+57$, $\Delta P1\Delta -48/+57$). Analysis of steady-state RNA synthesized by the $\Delta P1\Delta P2TATA$ construct revealed that transcription initiation occurred at multiple sites within a 30-bp region (Fig. 1D). A similar cluster of initiation sites has been observed in many other promoters lacking TATA sequences (Geng and Johnson 1993, and references therein). The synthesis of full-length RNA may be dependent on the presence of sequences immediately upstream of -48 , as further deletion up to -64 abrogated synthesis of full-length RNA. The transcription factor E2F has been shown to bind to the *c-myc* upstream region at -60 to -66 and be required for *c-myc* expression (for review, see Spencer and Groudine 1990).

These results indicate that sequences necessary to generate high levels of promoter-proximally paused transcription complexes are located at the core promoter region of the *c-myc* gene. In addition, these results suggest that the level of promoter-proximal pausing is not correlated with the amount of full-length RNA. [It is formally possible that these results are influenced by different stabilities of the tested RNAs because of their different 5' ends. However, the experiments presented

below (Fig. 2B) and the report that 5' sequences do not alter *c-myc* RNA stability (Bernstein et al. 1992) make this unlikely.]

Simple combination of Sp1 upstream activator and TATA/Inr sequences result in promoter-proximal pausing of RNA polymerase

The results described above indicate that sequences within the *c-myc* core promoter are necessary for the generation of high levels of paused RNA polymerase. To test whether the simple combination of core promoter elements with upstream activators is sufficient to generate promoter-proximal pausing, we used synthetic constructs (Smale et al. 1990) containing Sp1-binding sites as upstream activators combined with TATA- or Inr sequences. Consistent with results obtained with constructs containing the *c-myc* promoter, only very little promoter-proximal pausing was observed in the absence of a TATA and absence of an Inr consensus sequence (SP1-MG; Fig. 2). The insertion of the *c-myc* Inr-like region from position -12 to $+47$ (SP1-INR-MG) or the terminal-deoxynucleotidyl-transferase gene (Tdt)-Inr (SP1-Tdt-MG) or the TATA consensus sequence of the adenovirus major late promoter (SP1-TATA-MG) increased the amount of promoter-proximal RNA polymerase II complexes ~ 5 - to 10-fold. Similar to the results obtained with the *c-myc* gene, initiated complexes are very inefficient in elongation, and only little transcriptional activity is observed with run-on probes farther downstream. The deletion of upstream Sp1 sites (Δ SP1-TATA-MG; Fig. 2A) or replacement with GAL4-DNA-binding sites (GAL-TATA-MG; data not shown) results in very little promoter-proximal pausing (see below).

These results indicate that the recruitment of high levels of paused RNA polymerase II complexes requires two components: (1) core promoter elements and (2) upstream-activating sequences. Either element alone generates very little promoter-proximal pausing; however, both elements together mediate a higher-than-additive degree of transcriptional initiation. Thus, the formation of high levels of initiation-competent but elongation deficient RNA polymerase II transcription complexes is attributable to a synergy between upstream activators and proteins associated with core promoter elements.

Analyses of steady-state RNA by S1 analysis confirmed results described above that the generation of full-length RNA is independent of the level of promoter-proximal pausing. The construct without a consensus TATA or Inr sequence (SP1-MG) does not produce significantly less full-length RNA than the constructs containing elements of the *c-myc*, Tdt, or adenovirus major late core promoters displaying high promoter-proximal transcription (Fig. 2B).

Recruitment of elongation incompetent transcription complexes is a general property of transcriptional activators

The combination of TATA or Inr sequences with Sp1

Figure 2. A simple promoter containing a core promoter element in conjunction with Sp1 as upstream activator is sufficient to set up paused RNA polymerase. Synthetic promoter constructs containing 12Sp1 upstream sites and either no core promoter element (SP1-MG) or a TATA consensus sequence derived from adenovirus major late promoter (SP1-TATA-MG) or initiator sequences from -11 to +47 of the *c-myc* gene (SP1-INR-MG) or the *Tdt* gene (SP1-Tdt-MG) were combined with a hybrid reporter gene *myc-globin* (MG), and transfected into mouse L cells. (A) Nuclear run-on analyses of pools of stably transfected cells reveal that the formation of promoter-proximally paused transcription complexes can be observed in simple promoter constructs containing Sp1 upstream activators and core promoter elements. (B) Nuclease S1 analyses of total RNA isolated from transfected polyclonal lines. (Left) End-labeled, single-stranded DNA probes are specific for each construct. Single-stranded probes were generated from different templates with the same oligonucleotide. Probes extend from +132 to -335. (Right) Nuclease S1 analysis with a S1 probe homologous to SP1-INR-MG. This probe is only partially (from +38 to +132) homologous to transcripts derived from SP1-MG, SP1-TATA-MG, and SP1-Tdt-MG. The protected fragments of SP1-MG, SP1-TATA-MG, and SP1-Tdt-MG are of the same size and easier to quantify. Signals were counted with a PhosphorImager system and normalized to the amount of GAPDH. Although the level of RNA polymerase recruitment is increased 5-fold when TATA or Inr sequences are inserted, the steady-state levels of RNA are changed only 1.5-fold.

sites results in efficient recruitment of transcription complexes and a high level of RNA polymerases paused at promoter-proximal sites. Only a minor fraction of these complexes is detectable at sequences farther downstream. To investigate whether this phenomenon is limited to only Sp1-driven promoters or whether this is a general property of many transcriptional activators, we tested several other *trans*-activators for their ability to recruit RNA polymerase II complexes and influence the elongation competence of these transcription complexes. To simplify these analyses, we used the GAL4

fusion system where *trans*-activation domains of different upstream activators are fused to the DNA-binding domain of the yeast *trans*-activator GAL4. High levels of strongly activating proteins, however, may inhibit the transcription of genes through "squenching" (Gill and Ptashne 1988). This negative, repressing effect has been observed with GAL4-VP16 fusion proteins (Sadowski et al. 1988). For this reason, stable integration of such constructs in L cells might result in selection of cell clones that express only minor amounts of the *trans*-activator. Thus, we analyzed recruitment of RNA polymerase II

Figure 3. Recruitment of RNA polymerase II in synthetic promoter constructs after transient transfection. B78 cells were transfected with synthetic promoter constructs along with a β -gal gene driven by the CMV promoter as an internal control for transfection efficiency. Nuclear run-on assays confirm that in the absence of core promoter elements (SP1-MG; Fig. 2) or upstream activator elements (Δ SP1-TATA-MG), only very little promoter-proximally paused RNA polymerase is detected. Insertion of TATA sequence or the *c-myc* initiator region strongly increases the recruitment of RNA polymerases pausing within promoter-proximal regions.

and elongation efficiency after transient transfection into the murine melanoma cell line B78. In this cell line, expression of the endogenous *c-myc* gene is not detectable in nuclear run-on assays (data not shown). These cells reproduce faithfully the results obtained by stable transfections in L cells. Whereas constructs lacking a TATA box (SP1-MG) or upstream-activating sequences (Δ SP1-TATA-MG) show very little promoter-proximal pausing, high levels of RNA polymerase II loading was obtained with constructs where a TATA box or the *c-myc* Inr sequence is combined with Sp1 upstream activators (Fig. 3).

To analyze the influence of different *trans*-activation domains on initiation and elongation, we replaced Sp1-binding sites of our reporter construct SP1-TATA-MG with GAL4-binding sites. The resulting construct GAL-TATA-MG was transfected into B78 cells along with expression vectors coding for different *trans*-activation domains fused to the DNA-binding domain of the GAL4 protein; transcription of the cotransfected β -gal gene driven by the cytomegalovirus (CMV) promoter served as a control for transfection efficiency. When B78 cells were transfected with GAL-TATA-MG, CMV- β -gal, and the "empty" vectors containing no *trans*-activating sequences, a low signal with all probes was detected. (The source of this background signal is possibly derived from cryptic initiation sites in vector sequences.) Note that the run-on signals were not increased when the expres-

sion vector pSG(1-147) containing only the GAL4-DNA-binding domain was used, confirming that the GAL4-DNA-binding domain itself is not sufficient to confer transcriptional activation in mammalian cells [Keegan et al. 1986; see Discussion]. When the GAL4-SP1 hybrid proteins were coexpressed, the nuclear run-on signals obtained with promoter-proximal probes extending from +38 to +158 increased 10-fold (Fig. 4), indicating a high level of transcriptional initiation. A similar level of initiation was observed when GAL4 fusions of the *trans*-activators MyoD, CTF, and E1a were analyzed. GAL4-CCAAT/enhancer-binding protein (C/EBP), GAL4-p53, and GAL4-VP16 highly stimulated recruitment of RNA polymerase II complexes. All of these transcription activation domains, however, have in common that only a

Figure 4. Different *trans*-activators support the formation of paused RNA polymerase. The Sp1-binding sites of the SP1-TATA-MG construct (Fig. 3) were substituted with GAL4-binding sites. This construct was transfected into B78 cells along with plasmids expressing GAL4 fusion proteins and the CMV- β -gal control. (A) The transfected cells were analyzed in nuclear run-on assays. The locations of single-stranded run-on probes A_{GAL4}, and B-E are shown schematically. (B) Quantification of run-on signals. Nuclear run-on signals were counted in a PhosphorImager system, corrected for the cytidine content, and normalized to the signal obtained with the probe specific for β -gal transcripts. Note the different scale for the x-axis of GAL4-VP16, GAL4-p53, and GAL4-C/EBP *trans*-activated transcription.

fraction of initiated transcription complexes transcribe into regions farther downstream. Thus, we conclude that pausing of transcription complexes in promoter-proximal regions is a common property of promoters driven by many different *trans*-activators.

Enhancers influence elongation as well as initiation of transcription

Our results indicate that the simple combination of upstream activator and core promoter element (TATA or Inr sequences) is sufficient to recruit high levels of RNA polymerase II complexes. However, the increased level of initiation in the presence of core promoter elements compared with constructs lacking core promoters does not result in a linear increase in the amount of steady-state RNA (see Fig. 2). Similar to this result, it has been reported that the activity of synthetic promoter constructs containing SP1-binding sites did not vary in the presence or absence of a TATA box (Wang and Gralla 1991). However, a higher level of activity was achieved when enhancers were added to the constructs, and this enhancer-mediated higher level of activity was dependent on the presence of TATA sequences. These results suggest that the recruitment of transcription complexes through upstream activator and TATA is a necessary prerequisite for the function of enhancers. Thus, we were interested in determining whether synergistic acti-

vation of transcription relies on RNA polymerase II complexes already recruited through upstream activators and TATA or Inr elements. In this model, additional *trans*-activators might help to recruit factors that allow elongation-incompetent transcription complexes to escape into regions farther downstream. This would result in a higher proportion of transcription complexes detectable in nuclear run-on assays in regions farther downstream.

Elongation competence of transcription complexes could also be determined by the characteristics of upstream activators. Although every *trans*-activator tested supports the formation of paused polymerase, they might differ in their ability to recruit factors necessary for efficient elongation of transcription complexes. These differences would become evident in the ratio of transcription complexes found farther downstream relative to transcription complexes found in promoter-proximal regions.

To determine whether enhancer sequences or upstream activators influence the elongation competence of transcription complexes, we introduced the SV40 enhancer (SVE) downstream of the SP1-TATA-MG construct (Fig. 5A). This construct, SP1-TATA-SVE, was assayed after transient transfection of B78 cells. In addition, we compared elongation efficiencies of RNA polymerase II complexes recruited by the highly efficient upstream activator GAL4-VP16.

Quantitative analyses of the steady-state mRNA lev-

B

Figure 5. Enhancers greatly increase the amount of steady-state RNA, but not through suppression of the promoter-proximal pausing of RNA polymerase. (A) The construct GAL-TATA-MG was transfected along with plasmids for expression of GAL4-SP1 or GAL4-VP16 *trans*-activators into B78 cells. The construct SP1-TATA-MG was compared with SP1-TATA-SVE (containing the SV40 enhancer downstream of the MG reporter gene) after transient transfection. (B) Analysis of steady-state RNA in cells transfected with constructs shown in A. (C) Quantitative analysis of three independent nuclear run-on experiments. Location of probes within the transfected gene is shown in A. Quantifications were done with the PhosphorImager system.

C

els of transiently transfected SP1–TATA–SVE plasmids indicated a 20-fold increase in reporter mRNA compared with the construct lacking enhancer sequences (Fig. 5B). Similarly, synthetic reporter gene constructs activated by GAL4–VP16 synthesized an average of 80-fold more RNA than when activated by Sp1 in SP1–TATA–MG (Fig. 5B) or by GAL4–SP1 (data not shown). To analyze whether this increase was attributable to an increase in initiation or a release of RNA polymerase II complexes paused at promoter-proximal regions, we performed nuclear run-on assays. Each construct was tested at least three times in independent experiments. As shown in Figure 5C, the variation in these experiments was small. Nuclear run-on assays of cells transfected with SP1–TATA–MG and SP1–TATA–SVE revealed a 2.5-fold increase in RNA polymerase II recruitment by SVE, as indicated by the signal in the promoter-proximal probe B, covering the region between +36 and +158. GAL4–VP16 stimulated recruitment of RNA polymerase II about 3.5-fold compared with Sp1 (Fig. 5C). To test whether the presence of enhancer sequences or strong upstream activators affects the release of RNA polymerase II complexes paused at promoter-proximal sites, the ratio of run-on signals of the promoter-proximal probe B and the promoter-distal probe E was calculated (Fig. 5C). Constructs containing SVE sequences decreased the ratio of promoter-proximal to promoter-distal transcription complexes from 15:1 to 9:1, approximately twofold. The ratio for GAL4–VP16-activated transcription complexes was 5:1, corresponding to a threefold increase in transcription complexes in 3' regions relative to promoter-proximal regions.

Thus, not only do enhancer sequences and highly active upstream activators increase the amount of transcription initiation, they also increase the elongation competence of RNA polymerase II complexes as suggested by previous results (Madisen and Groudine 1994; Yankulov et al. 1994). In addition, these results suggest that different activators may differ in the extent of their effect on elongation as well as initiation.

Discussion

Sequences at the c-myc transcription initiation site are functionally equivalent to initiator elements

The high nuclear run-on signal observed within *c-myc* exon1 sequences is attributable to a high density of RNA polymerase II complexes in promoter-proximal regions. Recently, we have proposed that distinct promoter elements direct the assembly of RNA polymerase II complexes that differ in their elongation efficiency (Krumm et al. 1993). One such element has been described at the transcription initiation site of the HIV–LTR promoter and has been called inducer of short transcripts (IST). This element generates short transcripts within the HIV–LTR and has been shown to function even in the context of other RNA polymerase II or III promoters (Ratnasabapathy et al. 1990; Sheldon et al. 1993). Here, we provide evidence that sequences at the initiation site

of the *c-myc* gene also contribute to the recruitment of high levels of elongation-deficient transcription complexes. In addition, we show that high levels of promoter-proximally paused transcription are not only the consequence of the presence of initiator sequences, but can also be conferred by consensus TATA sequences.

Promoter-proximal pausing is a general feature of many promoters and may define a general rate-limiting step in transcription

The analysis of the sequence requirements for formation of paused polymerase in the *Drosophila hsp70* gene revealed that upstream binding sites for the constitutively expressed GAGA factor are important elements in setting up paused polymerase in the 5' region of the gene. Sequences around the transcriptional start site (–12 to +23) also seemed to influence pausing of RNA polymerase (Lee et al. 1992). Our results demonstrate that the simple combination of TATA sequences and upstream activators in synthetic promoter constructs is sufficient to generate a high level of promoter-proximally paused transcription complexes. Thus, this phenomenon is simply the result of synergistic interactions of regulatory factors and most likely occurs at many different promoters, rather than only at specialized promoters such as the *c-myc* or *hsp70* promoter.

The upstream activators Sp1, CTF, MyoD, and E1a and especially VP16, C/EBP, and p53 stimulate high levels of transcription complex formation, but only a few of these complexes actually transcribe regions farther downstream. Of the activators tested, GAL4–VP16 appeared to have the greatest positive effect on elongation competence. However, we did not observe complete suppression of promoter-proximal pausing by the VP16 *trans*-activation domain as reported previously (Yankulov et al. 1994). The most likely basis for these different observations is the different characteristics of the systems used in the analyses. For example, in our experiments (see Fig. 4), as well as in vitro (Wang et al. 1992; White et al. 1992), GAL4–VP16 highly stimulates recruitment of RNA polymerase II complexes to the promoter. However, in the system described by Yankulov et al., GAL4–VP16 does not increase transcription initiation significantly when compared with the GAL4–DNA-binding domain alone. Our experiments suggest that the acidic domain of VP16 enhances formation of the initiation complex ≥ 60 -fold higher than the GAL4–DNA-binding domain alone. Most of the transcription complexes, however, are paused at a promoter-proximal position.

Promoter-proximal pausing may define a common step in transcriptional activation, as it is observed with many promoters and different *trans*-activators. Although different *trans*-activators differ in the level of transcription complex recruitment, they are similar in that the highest amount of polymerase is detected at or close to the initiation site. Resistance to Sarkosyl indicates that these RNA polymerase II complexes are already initiated (Krumm et al. 1992). Thus, the major rate-limiting step in transcription in vivo seems to occur at a step after

transcription initiation. The polarity of RNA polymerase II density may be indicative of an antitermination mechanism, such as observed in prokaryotes at the λ pR' promoter, where the Q protein is required by RNA polymerase to escape out of the promoter-proximal pause at position +16 [for review, see Roberts 1993]. Similarly, efficient elongation of several *Drosophila* genes in vitro has been shown to depend on the presence of the positive elongation factor P-TEF. This factor has been shown to convert termination-prone transcription complexes into elongation-efficient transcription complexes [Kephart et al. 1992; Marshall and Price 1992].

In many aspects, promoter-proximal pausing resembles promoter clearance, which has been shown to influence transcription of genes in both prokaryotes and eukaryotes [Jacob et al. 1994 and references therein]. Recently, it has been suggested that the two general transcription factors TFIIE and TFIIH are not required for transcription initiation, but are necessary for promoter clearance [Goodrich and Tjian 1994; Maxon et al. 1994]. Thus, the high level of paused transcription complexes at promoter-proximal regions may be attributable to the lack of TFIIE and TFIIH at initiated complexes. The release of paused complexes may be regulated through recruitment of these general transcription factors. The interaction of these factors with the paused transcription complex may result in the phosphorylation of the carboxy-terminal domain (CTD) domain of RNA polymerase II. Different levels of CTD phosphorylation have been shown to be associated with paused and elongating RNA polymerase II complexes at the *hsp70* and other genes in *Drosophila* [Weeks et al. 1993; O'Brien et al. 1994]. In this context, it is important to note that some transcriptional *trans*-activators such as GAL4-VP16 and GAL4-p53 have been reported to bind to TFIIH [Xiao et al. 1994].

Alternatively, promoter-proximal pausing may be attributable to abortive initiation mechanisms. This model differs from the antitermination model discussed above in that pausing or terminating transcription complexes may not be converted into "readthrough" transcription complexes; rather, the generation of full-length RNA requires de novo initiation where distinct promoter elements necessary for efficient elongation of the transcription complex recruit factors to the initiation or early elongation complex [see Krumm et al. 1993 for further discussion].

Transcriptional synergy functions at both levels, initiation and elongation

High mRNA levels are obtained through synergistic interaction of multiple upstream activators. Transcriptional synergy refers to the greater than additive increase in transcription when more than one type of *trans*-activator is present or when additional binding sites are added. The basic mechanism underlying synergism remains unknown. The mechanism could be attributable to cooperativity in binding of *trans*-activators. Cooperative binding is not always observed on naked DNA tem-

plates and in some cases requires DNA packaged into chromatin [Workman et al. 1991; Croston et al. 1992; Chang and Gralla 1994]. However, synergistic activation has been observed under conditions where the binding sites of the activator are saturated [Carey et al. 1990; Lin et al. 1990]. This result supports the idea that synergizing *trans*-activators may catalyze different steps during transcription complex assembly [for review, see Herschlag and Johnson 1993]. For example, the ability of the *trans*-activator VP16 to interact with both TFIID and TFIIB may explain the synergistic response obtained with promoters containing more than one binding site. One molecule could catalyze the binding of TFIID, whereas the other molecule could increase the efficiency of TFIIB recruitment or stabilize its binding. Here we demonstrate a different level of transcriptional synergy. Whereas the upstream activator stimulates the recruitment of RNA polymerase II complexes, additional *trans*-activators recruited through enhancer sequences confer elongation efficiency to the transcription complexes. Thus, transcriptional synergy may be obtained by facilitating sequential steps at initiation and elongation of transcripts.

In a recent study, it has been shown that multiple activators increase the amount of TFIIE loaded into the transcription complexes [Choy and Green 1993]. As mentioned above, TFIIE as well as TFIIH have been suggested to be essential for promoter clearance rather than for formation of functional initiation complexes. Thus, it is possible that RNA polymerase II complexes recruited through proximal upstream activators are devoid of TFIIE or TFIIH, leading to inefficient promoter clearance. During the nuclear run-on assay, however, these polymerases are released to transcribe regions farther downstream, even in the absence of above mentioned "clearance" factors.

The recruitment of transcription complexes through interaction of the Sp1 upstream activator with elements of the core promoter is synergistic itself. The combination of upstream activator and TATA or Inr sequence recruit much higher levels of transcription complexes than either element alone. These complexes are very inefficient at the elongation phase of transcription. Additional factors seem to be required to render these complexes elongation efficient or to recruit elongation-efficient RNA polymerase II complexes de novo.

Elongation rate as an important mechanism for gene regulation

The increase in the level of full-length RNA in the presence of upstream activators and enhancers does not correspond to the observed modest increase in transcriptional initiation. Although efficient *trans*-activators and enhancers can increase the amount of transcription complexes detected farther downstream relative to the amount of RNA polymerase II complexes at the promoter (this study; Madisen and Groudine 1994; Yankulov et al. 1994), the much higher level of full-length RNA is not simply explained by the relief of pausing near the

promoter. The release of promoter-proximally stalled RNA polymerase II complexes would be expected to result in a proportional increase of run-on signals detected with downstream probes. However, in most cases, this has not been observed. Formally, this discrepancy could be explained by limitations of the run-on assay, including the possible failure of downstream RNA polymerases to resume transcription in this assay. Alternatively, enhancers/activators may increase the rate of transcription complex elongation by decreasing or suppressing pausing of RNA polymerase II complexes once they are out of the promoter-proximal region. Transcription complexes that pass through the gene at a very high rate would be difficult to detect, as the average time spent transcribing the template would be reduced drastically. Thus, although strong upstream activators and enhancers examined in this study confer modest increases in initiation and release of polymerase from the promoter-proximal pause site, we propose that they also influence gene activity through modulating the elongation rate of the transcription complex.

Elongation rates are influenced by different mechanisms. For example, nucleosomes have been described to impede transcriptional elongation (Izban and Luse 1991). Recently, HMG-14, a nonhistone chromosomal protein, has been reported to increase transcriptional elongation about fourfold in *in vitro* transcription reactions on nucleosomal templates (Ding et al. 1994). Although HMG-14 and other nonhistone proteins are abundant nuclear proteins, some are found only in transcriptionally active regions (Weisbrod et al. 1980). In *in vitro* transcription experiments, the general transcription factor TFIIF has been reported to function at the level of elongation and was proposed to act as a processivity factor through suppression of pause sites of polymerase complexes, thereby increasing the rate of transcription (Bengal et al. 1991). Regulation at the level of elongation rate has also been suggested to be responsible for modulation of RNA levels from the HIV-LTR promoter (Kato et al. 1992). In this study, Tat has been described as a factor cooperating with and stimulating the activity of the elongation factor TFIIF.

A general model for the postinitiation control of transcription

With the evidences available from several studies, the following model may describe the regulation of transcription at the level of elongation (Fig. 6). High levels of RNA polymerase II complexes are recruited through the synergistic action of upstream activator and TATA/Inr sequences. Initiated transcription complexes pause at or immediately downstream of the transcription initiation site. Upon modification, transcription complexes are released into downstream regions. This modification may involve the general transcription factors TFIIE and TFIIH, both suggested to be required for promoter clearance. The interaction of promoter-proximal transcription complexes with these general transcription factors may result in (re)phosphorylation of the CTD of RNA

polymerase II. Alternatively, as discussed above, the modification of the transcription complex may occur before initiation of transcription. Partly assembled RNA polymerase complexes lacking the general transcription factors TFIIE and TFIIH or others may initiate transcription, however, pause and finally terminate within promoter-proximal regions. Only completely assembled transcription complexes generate full-length RNA. An additional level of transcriptional control may be exerted through modulation of elongation rates. This mechanism may function at the level of chromatin structure/accessibility and factors suppressing idling of RNA polymerase II complexes at intrinsic pause sites.

The proposed model could account for many observations made in several systems where transcriptional synergy has been observed. For instance, transcriptional activation of the HIV type 1 promoter involves synergy between the viral Tat protein and Sp1 (Southgate and Green 1991; Kamine and Chinnadurai 1992). Whereas the deletion of NF- κ B sites results only in a modest decrease in transcription, deletion of Sp1-binding sites results in a drastic reduction of Tat *trans*-activation (Kamine et al. 1991). The HIV core promoter (−43 to +80) cannot be induced by Tat even in the presence of the *trans*-activation response (TAR) sequence. Moreover, the simple addition of synthetic upstream activator motifs (Sp1, Oct, and others) confer Tat responsiveness (Berkhout and Jeang 1992). In addition, *trans*-activation by Tat requires the HIV-TATA motif (Garcia et al. 1989; Olsen and Rosen 1992; Lu et al. 1993). These experiments could be interpreted to confirm the simple model of promoter structure proposed in this study, where the combination of TATA sequences and upstream activators are necessary to recruit high levels of transcription complexes. In the absence of those promoter elements, the promoter is still functional and generates basal levels of full-length RNA; however, this promoter cannot respond to induction signals required to recruit transcription complexes highly efficient in elongation. In the case of the HIV-LTR, this signal would be provided by or mediated through the binding of Tat protein to the TAR. In general, for most promoters, *trans*-activators recruited by enhancers or upstream sequences, or both, may determine the elongation competence of the transcription complex.

Materials and methods

Plasmids and M13 constructs

The pAP1 *c-myc* construct from which the P1TATA box through the P1 cap site are deleted has been described previously (Spencer et al. 1990). This construct contains an intact P2 promoter and is used in this study as the “wild-type” promoter. To construct pAP1ΔP2TATA, the *Xho*I-*Xba*I fragment of pAP1 was substituted with the same fragment of pAP2TATA (Spencer et al. 1990) containing a deletion of the sequence TATA upstream of the *c-myc* P2 transcription initiation site. Core promoter deletions were generated by cutting linker-scanner mutants pAP1LS12, pAP1LS7, and pAP1LS4 (Meulia et al. 1992) with *Eco*RI and *Sap*I, and religating the ends after filling in with

Figure 6. Model describing transcriptional regulation at the level of promoter-proximal pausing. (A) Preinitiation complex is formed through interaction of upstream activator with general transcription factors TFIID and TFIIB. (B) After initiation the transcription complex is paused at sequences around +30, possibly through interaction of the transcribing complex with TFIID through the CTD domain with upstream activators. (C) Recruitment of TFIIE and TFIIH through upstream activators results in phosphorylation of the CTD and disruption of the TFIID-CTD interaction. Additional factors (?) may be loaded to render the transcription complex resistant to intrinsic pausing signals further downstream, thereby increasing the elongation rate. (D) RNA polymerase complex leaving the promoter to generate full-length RNA. (E) The failure to recruit TFIIE and TFIIH or additional factors (?) may result in termination of transcription.

T4 polymerase. Synthetic promoter constructs containing Sp1-binding sites alone (plasmid III; Smale et al. 1990), or in addition to a TATA box derived from the adenovirus major late promoter (plasmid V; Smale et al. 1990), or the initiator region of the Tdt (plasmid VI; Smale et al. 1990). To insert sequences of the *c-myc* transcription initiation site, plasmid III was cut with *EcoRI* and *SmaI* and the *EcoRI*-*NaeI* fragment (sequences -11 to +47 relative to the P2 promoter) of the linker-scanner plasmid pΔPILS12 (Meulia et al. 1992) was inserted. As a reporter gene, the *HindIII*-*MscI* fragment of the fusion gene *myc*-globin (MG) was cloned into the *HindIII* and *PvuII* site of the synthetic promoter constructs to obtain SP1-MG, SP1-TATA-MG, SP1-Tdt-MG, and SP1-Inr-MG. ΔSP1-TATA-MG was generated through excision of the *HpaI*-*BglII* fragment containing the binding sites for upstream activator Sp1. SP1-TATA-SVE was constructed through insertion of a *HincII* fragment of pCAT-control (Promega) containing the SV40 enhancer into the *AatII* site, downstream of the MG reporter of SP1-TATA-MG. GAL4 promoter construct GAL4-TATA-MG was constructed through excision of Sp1-binding sites by *HpaI* and *BglII*, and insertion of a 79-bp *SmaI*-*SauIII*A fragment of pSH384 (gift of S. Hahn) containing three GAL4-binding sites.

GAL4 expression plasmids GAL4(1-147), GAL4-VP16, GAL4-Sp1, GAL4-P53, and GAL4-CTF were provided by C. Chang and J. Gralla, GAL4-E1a by M. Green, GAL4-MyoD by H. Weintraub.

Cell lines and transfection

The mouse L cell fibroblast line and the melanoma cell line B78 were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% bovine calf serum and 2 mM glutamine.

Mouse L cells were transfected by electroporation as described (Krumm et al. 1992). B78 cells were transfected by the calcium phosphate precipitation method (Sambrook et al. 1989). For a 15-cm dish, a total of 27.5 μg plasmid DNA was used. Briefly, a 25-μg of reporter plasmid (or a 18 μg of GAL4-TATA-MG in addition to a 7-μg GAL4 *trans*-activator expression plasmid) were combined with a 2.5-μg CMV β-gal plasmid as a transfection control and combined with 660 μl 0.1× TE [1 mM Tris-HCl (pH 8), and 0.1 mM EDTA], 750 μl 2× HBS (280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄·2H₂O, 12 mM dextrose, 50 mM HEPES). After addition of 93 μl 2 M CaCl₂, the solution was added to a 15-cm dish containing 2×10⁶ cells. After incubation overnight, the cells were washed with Tris and fed with fresh DMEM medium. Twenty-four hours later, cells were harvested and nuclei or RNA were prepared.

RNA and DNA analyses

Total RNA for S1 protection analyses was isolated from cells with RNazol (Cinna/Biotechx) according to the manufacturer's protocol. 5' End-labeled probes were generated by extension of

an oligonucleotide, phosphorylated with T4-kinase and [γ - 32 P]ATP, in a 14-cycle PCR reaction on linearized double-stranded plasmid DNA (5 μ g). End-labeled single-stranded DNA was isolated on a 6% acrylamide gel. The mouse GAPDH probe is described in Madisen and Groudine (1994). Hybridization of single-stranded probe with total RNA was performed as described (Meulia et al. 1992). Southern analyses were done as described (Sambrook et al. 1989).

Nuclear run-on analyses

Nuclear run-on assays were done as described (Krumm et al. 1992) with the following modifications: After DNase I treatment of nuclei and proteinase K treatment for 20 min at 55°C, RNA was purified with two phenol-chloroform (1:1) extractions. Labeled RNA was precipitated with three volumes of ethanol, resuspended in TE, and separated from unincorporated nucleotides by use of a Sephadex G50 column. Run-on assays with nuclei of transiently transfected B78 cells were performed with [α - 32 P]CTP. Control experiments with HL60 cells and transfected L cells revealed no difference whether [α - 32 P]UTP or [α - 32 P]CTP was used as the label. M13 probes A–E of regions of the *c-myc* gene as hybridization probes correspond to P_o, SR, NS, RS, and SA probes described in Krumm et al. (1992). The probes A_{sp1} used to detect upstream transcription in Sp1-driven promoters was generated through subcloning of a 0.3-kb *Hpa*I–*Bgl*III fragment into M13 mp18 and M13 mp19. The A_{Gal} probe detecting upstream transcripts in the GAL4–TATA–MG construct was excised from pSH384 with *Sma*I (see above).

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Note added in proof

After this paper was in press, Zhou and Sharp [*EMBO J.* (1995) 14: 321–328], investigating the transcriptional activation of the HIV-LTR in vitro, reported results supporting the view that upstream activators in combination with TATA sequences recruit high levels of elongation-deficient transcription complexes, and additional signals are required to render these complexes elongation efficient.

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