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Stability of *Drosophila* RNA Polymerase II Elongation Complexes In Vitro

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We show that nuclear extract from *Drosophila* K_c cells supports efficient elongation by RNA polymerase II initiated from the actin 5C promoter. The addition of 0.3% Sarkosyl, 1 mg of heparin per ml, or 250 mM KCl immediately after initiation has two effects. First, the elongation rate is reduced 80 to 90% as a result of the inhibition of elongation factors. Second, there is an increase in the amount of long runoff RNA, suggesting that there is an early block to elongation that is relieved by the disruptive reagents. Consistent with the first effect, we find that the ability of factor 5 (TFIIF) to stimulate the elongation rate is inhibited by the disruptive agents when assayed in a defined system containing pure RNA polymerase II and a dC-tailed template. The disruptive agents also inhibit the ability of DmS-II to suppress transcriptional pausing but only slightly reduce the ability of DmS-II to increase the elongation rate twofold. The pause sites encountered by RNA polymerase II after initiation at a promoter and subsequent treatment with the disruptive reagents are also recognized by pure polymerase transcribing a dC-tailed template. It has been suggested that 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole inhibits RNA polymerase II during elongation, but we find that the purine nucleoside analog has no effect on elongation complexes containing RNA over 500 nucleotides in length or on the action of factor 5 or DmS-II in the defined system.

Control of transcription is of vital importance in the regulation of eucaryotic gene expression. Transcription is a complex, multistep process that can be functionally divided into three distinct phases: initiation, elongation, and termination. Initiation encompasses the location of a promoter by RNA polymerase II, the formation of the first phosphodiester bond, and the escape from abortive initiation. During the elongation phase, RNA polymerase II catalyzes the processive addition of ribonucleotides to the 3′ end of the growing RNA chain until specific attenuation or termination signals are encountered. Finally, transcription is terminated and polymerase is released from the DNA template. An understanding of initiation of transcription by RNA polymerase II, as well as the functions of many of the factors involved, has been facilitated by a combination of in vivo and in vitro studies. However, the elongation and termination phases of transcription are not as well understood.

There is an increasing number of genes for which expression appears to be controlled at the level of elongation (71). Elongation is clearly involved in the control of expression of the proto-oncogenes c-*myc* (42, 50, 56, 72, 76), c-*myb* (1, 51), and c-*fos* (12, 63). Many viruses, such as adenovirus (31, 57, 65), simian virus 40 (30, 58), and minute virus of mice (33), have premature blocks to transcription elongation. Human immunodeficiency virus has a block to elongation that can be relieved by the viral Tat protein (34, 35, 67, 73). The adenosine deaminase genes in humans and mice have blocks to elongation that are modulated in order to vary adenosine deaminase mRNA levels in cells (9, 10, 36, 40, 48). In *Drosophila melanogaster*, many genes contain RNA polymerase II molecules arrested in the process of elongation (59, 60), suggesting that control of elongation may be a general mechanism used to modulate transcriptional activity.

Two different experimental approaches have been used to study the postinitiation phases of transcription by RNA polymerase II in vitro. Because the transcription of promoter-containing DNA templates requires numerous initiation factors present in impure protein fractions, a system was developed in which pure RNA polymerase II initiates at the end of a dC-tailed DNA template (23). The dC-tailed template system has been useful in studying sequence-specific pausing and termination by RNA polymerase II (26, 28, 29, 55, 70) and has been used in the identification, purification, and characterization of protein factors which affect elongation by RNA polymerase II (26–28, 46, 47, 52, 54, 68–70). A second experimental approach uses templates with physiological promoters and crude extracts that support initiation of transcription. These systems have been used to examine the transition into elongation and some aspects of the effects of factors on elongation (2–4, 7, 14, 22, 31, 32, 37–39, 61, 75).

The first RNA polymerase II elongation factor described, S-II, was purified from mouse cells as an activity that stimulated transcription in the presence of manganese ions (66). This factor has been shown to be involved in the elongation phase of transcription (21, 49, 52, 54, 68). Factors similar to S-II have been identified in other organisms (62, 69). The mammalian factor S-II (also called TFIIS) and its *Drosophila* homolog, DmS-II, suppress transcriptional pausing at specific DNA sequences in vitro (49, 52, 69). The features of the paused transcription complex that are recognized by S-II and the mechanism by which the factor functions are unknown. The cDNAs encoding mouse S-II (20) and the *Drosophila* homolog, DmS-II (41), have been isolated. Comparison of their sequences suggests that the factor has two conserved domains separated by a central serine-rich region (41), although the functional significance of these domains of homology is not known.

One factor that stimulates elongation by RNA polymerase II has also been shown to be utilized in the initiation process. Factor 5 was first identified as an activity that was required for initiation in vitro which could also stimulate elongation by RNA polymerase II (46). *Drosophila* factor 5 and its

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mammalian counterpart, TFIIF or RAP 30/74, have a high affinity for purified RNA polymerase II (5, 15, 47, 53). However, the *Drosophila* factor loses this affinity during elongation (47). Kinetic studies indicate that factor 5 interacts briefly with the paused transcription complex and then dissociates from the elongation complex as it reenters active elongation (47). The role that factor 5 plays in initiation and the mechanism by which it stimulates elongation are unknown.

Two other eucaryotic factors that affect elongation by RNA polymerase II have been identified. TFIIX was identified in HeLa cell extract and was found to stimulate the rate of elongation similarly to TFIIF and factor 5 (3, 32). This factor is not required for initiation of transcription (3) and has not yet been purified to homogeneity. YES, a factor purified from yeast cells, was found to stimulate elongation by RNA polymerase II (8). This 116-kDa yeast protein was specific for RNA polymerase II and has properties most similar to those of *Drosophila* factor 5 (8), although it is not known whether this factor also plays a role in initiation.

Understanding how elongation is controlled requires the use of in vitro systems that mimic the in vivo situation. Although in vitro studies have led to a more detailed understanding of the initial events in eucaryotic transcription, relatively little is known about the incorporation of elongation factors into the transcription complex. In this study, we used two in vitro approaches to investigate the interaction of transcription factors with elongating polymerase. We used unfractionated *Drosophila* K_c cell nuclear extract to drive initiation from a physiological promoter and then examined the stability of the resulting elongation complexes to Sarkosyl, heparin, 250 mM KCl, or 5,6-dichloro-β-D-ribofuranosylbenzimidole (DRB). We also used an assay which allows purified RNA polymerase II to initiate at a discrete site without requiring specific initiation factors (23). Our results indicate that heparin, Sarkosyl, or 250 mM KCl inhibits the action of elongation factors forcing RNA polymerase II which has initiated from a promoter to transcribe the template unaided by elongation factors. We have also uncovered evidence that many transcription complexes experience an early block to elongation that can be relieved by heparin, Sarkosyl, or high salt.

MATERIALS AND METHODS

Chemicals. Ribonucleoside triphosphates were obtained from Pharmacia LKB Biotechnology Inc. [α-^{32}P]CTP (650 Ci/mmol) was purchased from ICN pharmaceuticals. All other materials were reagent grade.

DNA templates. A2(R/H) was constructed by subcloning a 3,860-bp *Eco*RI- and *Hin*dIII-digested fragment containing the promoter of the *Drosophila* actin gene, Act 5C (17), into pSP73 (Promega Biotec). Transcription of this construct after digestion with *Sal*I yields an 1,100-nucleotide runoff transcript. A2(R/P) consists of a 3,030-bp *Eco*RI- and *Pst*I-digested fragment containing the Act 5C promoter subcloned into pSP73. Transcription of this construct after digestion with *Eco*RI yields a 3,000-nucleotide runoff transcript consisting of the first 460 bp of Act 5C and all of pSP73. A2(A/H) was constructed by subcloning the 1.3-kb *Ava*II-*Hin*dIII fragment from Act 5C into pSP73. dC-A2 and dC-3500 were obtained by digesting this construct with *Pst*I and tailing the two fragments generated (500 and 3,500 bp) with dCTP and terminal transferase (45). The dC-A2 was digested with *Hae*III to remove the dC tail at one end. This generated a tailed DNA fragment containing the first 460 bp of Act 5C preceded by 21 nucleotides. The 3,500-bp fragment, dC tailed at both ends, contains sequences from 500 to 1,300 bp downstream from the start site of transcription of Act 5C and all of pSP73.

Extracts and transcription factors. Growth of K_c cells and preparation of nuclear extract have been described elsewhere (46). *Drosophila* RNA polymerase II was isolated from embryos as previously reported (46). Factor 5 and DmS-II were purified from K_c cell nuclear extract as previously described (46, 47).

Transcription using Kc cell nuclear extract. A pulse-chase protocol was used in which the template DNA was preincubated with the extract, 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES; pH 7.6), and 5 mM $MgCl_2$ for 10 min at 23°C. Nucleotides, including [α-^{32}P]CTP, were added to start the pulse; 15 to 90 s later (as indicated in figure legends), cold CTP was added to initiate the chase. Reactions were stopped after the indicated chase times. During the pulse, 10-μl reaction mixtures contained the following: 20 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 600 μM each GTP, UTP, and ATP, 5 μCi of [α-^{32}P]CTP (about 1 μM CTP), 60 mM KCl, 60 or 240 ng of DNA template (as indicated), and 4 μl of K_c cell nuclear extract. Unlabeled CTP was then added to bring the total concentration of CTP to 1.2 mM and the final reaction volume to 12 μl. Reaction mixtures containing high KCl, Sarkosyl, or heparin were supplemented with these reagents either at the beginning of the chase or 30 s later, as indicated. Reactions were stopped by adding 200 μl of ice-cold stop solution (1% Sarkosyl, 50 mM Tris [pH 8.0], 50 mM EDTA, 100 mM NaCl, 100 μg of tRNA per ml). The reaction mixtures were phenol extracted; the nucleic acids were ethanol precipitated, washed with 70% ethanol, dried, and analyzed by gel electrophoresis.

Transcription of the dC-tailed template. dC-tailed template reactions were similar to those previously described (8). Reaction mixtures (13 μl) contained 20 mM HEPES (pH 7.6), 600 μM each GTP, UTP, and ATP, 5 mM $MgCl_2$, 60 to 75 mM KCl, 5 μCi of [α-^{32}P]CTP, 300 μg of bovine serum albumin per ml, 60 ng of DNA, 10 U of purified *Drosophila* RNA polymerase, and purified transcription factors as specified. Continuous-labeling reaction mixtures also contained 30 μM CTP. For pulse-chase reactions, the CTP was raised to 1.2 mM during the chase. In all cases, DNA was preincubated with polymerase for 5 min at 23°C under limiting UTP conditions (no added UTP) to allow association of polymerase and template. Reactions were started by addition of UTP, KCl, and water. In the continuous-labeling experiments with factor 5, the factor was added with the UTP and elongation continued for 5 min. For pulse-chase experiments with DmS-II, the factor was added coincident with the cold CTP at the beginning of a 10-min chase. Reaction products were isolated and analyzed as described above for transcription using K_c cell nuclear extract.

Gel electrophoresis. For polyacrylamide gel electrophoresis, samples were resuspended in 6 μl of 0.25× TBE (1× TBE is 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA)–8 M urea, heated for 5 min at 85°C, and analyzed in 6% acrylamide–6 M urea–1× TBE gels as described by Price et al. (46). Denaturing agarose gel electrophoresis was performed by resuspending samples in 50% formamide–2.2 M formaldehyde–1× morpholinepropanesulfonic acid (MOPS) buffer (20 mM MOPS [pH 7.0], 5 mM sodium acetate, 1 mM EDTA) and heating the mixture at 85°C for 5 min. Samples were resolved in a 1.85% agarose gel containing 2.2 M formaldehyde and 1× MOPS buffer.

High-resolution mapping of pause sites. DNA sequencing

reactions were performed by the dideoxy method, using primers with 5′ ends similar to the 5′ ends of transcripts to be analyzed. One primer (ATCACTACCGTTTGAGTTC) corresponded to nucleotides +1 to +19 of Act 5C. The other primer (GCAGGCATGCAAGCTT) began 3 nucleotides upstream of the single-strand/double-strand junction of the tailed end of dC-A2. The 5′ end of this primer was chosen because RNA polymerase II begins transcription of tailed *Pst*I fragments at this location (70). Sequencing reaction products labeled with [α-^{35}S]dATP were electrophoresed with appropriate [α-^{32}P]CTP-labeled transcripts on denaturing 6% polyacrylamide sequencing gels. A correlation of RNA transcript size with DNA sequence was made by comparing the pattern of transcripts generated under CTP-limiting conditions with dC residues observed in the DNA sequence. In the analysis of the RNA synthesized from the Act 5C promoter, the comparison of the pulsed RNA (0-min chase) sequence with the DNA sequence indicated that a 2.5-nucleotide shift was necessary to align the RNA and DNA sequences. For analysis of pausing by purified RNA polymerase II on dC-A2, the RNA synthesized during an extended chase with limiting CTP (3 μM) was compared with the DNA sequence. A shift of 1.5 nucleotides was required to align the sequence with the RNA. Three separate gel analyses to determine the 3′ ends of transcripts gave identical results.

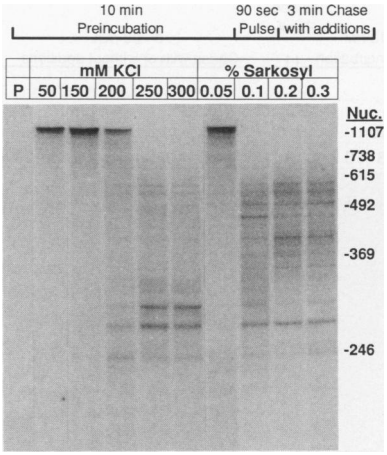

FIG. 1. Titration of the effects of salt or Sarkosyl on elongation. Transcription complexes were formed on the Act 5C promoter by incubating *Sal*I-digested A2(R/H) with K_c cell nuclear extract as outlined in Materials and Methods. After a 90-s pulse, reactions were chased for 3 min in the presence of increasing concentrations of KCl or Sarkosyl. Transcription reactions were stopped and analyzed by denaturing 6% polyacrylamide gel electrophoresis as specified in Materials and Methods. Final concentrations of KCl and Sarkosyl are indicated above the gel. Lane P, transcription reactions stopped after the pulse. The sizes of DNA markers are given in nucleotides (Nuc.).

RESULTS

We have previously identified two *Drosophila* factors, factor 5 and DmS-II, which affect the elongation properties of RNA polymerase II in a completely defined system (46, 47, 69). In an effort to study their involvement in elongation by RNA polymerase II which has initiated from a promoter, we sought to determine the properties of elongation complexes formed in unfractionated K_c cell nuclear extract. This extract contains RNA polymerase II and factors necessary for specific initiation (44, 46) as well as factors which affect elongation. Preliminary evidence suggested that in the presence of the extract, the elongation rate of RNA polymerase II initiated from a promoter was quite high. We began our studies by examining elongation by RNA polymerase II under a variety of conditions.

Effects of salt, Sarkosyl, or heparin on elongation complexes. We first investigated the effects of increasing concentrations of KCl or Sarkosyl on RNA polymerase II elongation complexes formed after initiation at the *Drosophila* Act 5C promoter. After a 10-min preincubation, transcription was initiated during a 90-s pulse-labeling. Elongation continued under chase conditions in the presence of increasing concentrations of either KCl or Sarkosyl. The labeled RNA was then analyzed by denaturing polyacrylamide gel electrophoresis (Fig. 1). After the 90-s pulse, elongation complexes have transcribed less than 150 nucleotides because of limiting CTP concentration (Fig. 1, lane P). Elongation was followed by performing a 3-min chase with nonlimiting nucleoside triphosphates (NTPs). Elongation in 50 mM KCl gave rise to an 1,100-nucleotide runoff transcript in less than 3 min. Transcripts shorter than full-length runoff appeared when elongation took place in 200 mM KCl. KCl concentrations of 250 mM or higher caused accumulation of the short transcripts and did not allow the production of runoff transcripts in 3 min.

A similar protocol demonstrated that Sarkosyl also affected efficient elongation (Fig. 1). Although elongation was essentially unaffected by the presence of 0.05% Sarkosyl, the addition of Sarkosyl to a final concentration of 0.1% led to the appearance of short transcripts. Increasing the Sarkosyl concentration in the chase to higher than 0.1% had no additional effect. Heparin was also found to inhibit elongation and had a maximal effect at 1 mg/ml (data not shown).

To determine whether salt, Sarkosyl, or heparin caused a reduction of the elongation rate of complexes formed after initiation from the Act 5C promoter, we analyzed transcripts produced by transcription complexes during the elongation phase in the presence or absence of these chemical agents. A pulse-chase protocol was used (see diagram in Fig. 2). Elongation was carried out in K_c cell nuclear extract with no addition or in the presence of 250 mM KCl, 0.3% Sarkosyl, or 1 mg of heparin per ml. Elongation was followed by stopping reactions at 1, 3, 10, or 30 min after the beginning of the chase and analyzing the transcripts generated by polyacrylamide gel electrophoresis (Fig. 2).

As expected, transcription reactions performed at 60 mM KCl exhibited a relative lack of paused elongation complexes and were sensitive to 2 μg of α-amanitin per ml (Fig. 2). The accumulation of runoff transcript was not complete in 3 min, although some polymerase molecules were able to reach the 1,100-nucleotide runoff in 1 min (longer exposure of gel; data not shown). Elongation in the presence of 250 mM KCl, 0.3% Sarkosyl, or 1 mg of heparin per ml led to enhanced pausing of the transcription complexes at specific sites on the DNA template (Fig. 2). Pausing rather than premature termination is indicated because most of the transcripts were chased into longer species. A comparison of transcripts generated indicates that RNA polymerase II recognizes similar sequence-specific pause sites in the presence of salt, Sarkosyl, or heparin. However, variation in the intensity of some bands can be seen when different chemical agents are used. To quantitate the effect that the chemical reagents had on transcription complexes, we determined maximum elongation rates in untreated K_c cell nuclear

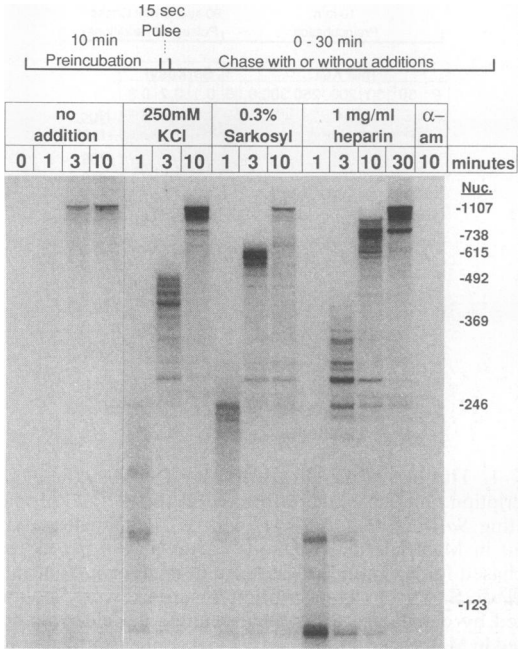

FIG. 2. Evidence that Sarkosyl, heparin, or 250 mM KCl enhances pausing and decreases the elongation rate. Transcription reactions using K_c cell nuclear extract and A2(R/H) template cut with *Sal*I were performed by using a pulse-chase protocol as described in Materials and Methods. Reactions were pulse-labeled for 15 s and then chased with or without the addition of 250 mM KCl, 0.3% Sarkosyl, or 1 mg of heparin per ml, as indicated. Aliquots (12 μl) of a larger reaction mixture were taken at the times indicated and resolved by 6% denaturing polyacrylamide gel electrophoresis as outlined in Materials and Methods. The exposure time of lanes corresponding to transcription reactions using untreated extract is twice that of lanes for reactions containing additions. Lane α-am, transcription reaction with the addition of α-amanitin to a final concentration of 2 μg/ml during the preincubation. The sizes of DNA markers are given in nucleotides (Nuc.).

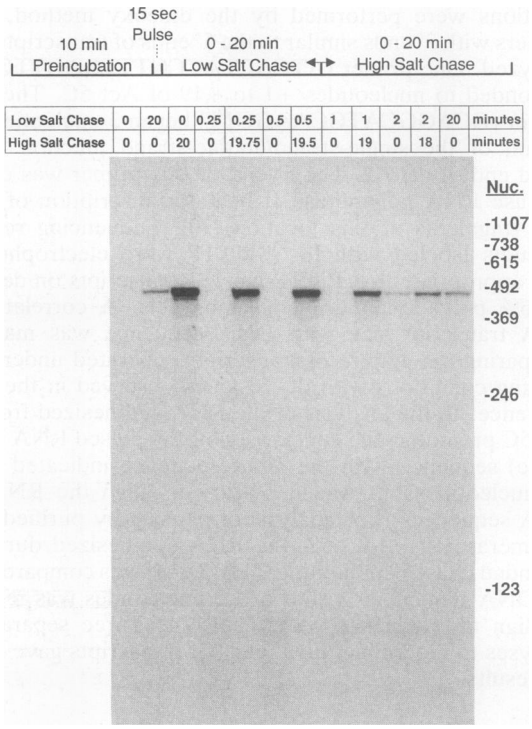

FIG. 3. Time dependence of 250 mM KCl stimulation of runoff transcription. Reactions were carried out with a modified pulse-chase protocol (see Materials and Methods). The diagram above the gel indicates the timing of the steps of the reaction. The pulse was followed by a chase in either 60 mM (low salt) or 250 mM KCl (high salt). Some reactions were shifted to high salt after the indicated times. Total reaction volume was 12 μl for chases done at a single salt concentration or 14 μl for reactions in which chases were done at both low and high salt. The total chase time for all reactions (except the 0 chase control) was 20 min. Transcripts were analyzed in a 6% acrylamide–TBE–urea gel. The positions of DNA markers are indicated. The runoff transcript is 460 nucleotides (Nuc.) long.

extract or in the presence of 250 mM KCl, 0.3% Sarkosyl, or 1 mg of heparin per ml (Table 1). RNA polymerase II achieved a maximum elongation rate of 900 to 1100 nucleotides per min in untreated K_c cell nuclear extract at 23°C. This is similar to the reported in vivo rate (74). Many polymerase molecules do not reach the 1,100-nucleotide runoff even in 3 min (Fig. 2), which suggests either a wide distribution in elongation rates for individual complexes or a slow step in the release of the complexes from promoter proximal positions. The latter possibility is supported by experiments described later and in the accompanying report (40a). Elongation in the presence of 250 mM KCl, Sarkosyl, or heparin slowed to between 100 and 200 nucleotides per min, which is similar to the rate determined for RNA polymerase II on a dC-tailed template under similar ionic conditions (70).

TABLE 1. Maximum elongation rates in untreated or treated extracts[a]

Addition during chase	Elongation rate (nucleotides/min)
None	1,011 ± 11
250 mM KCl	188 ± 20
0.3% Sarkosyl	205 ± 9
1 mg of heparin per ml	99 ± 10

[a] Elongation rates in untreated nuclear extracts were measured by using a template generating a 3,000-bp runoff under conditions similar to those used for Fig. 3. Elongation rates in the presence of high KCl, Sarkosyl, or heparin were measured from experiments identical to those in Fig. 2. Rates were calculated by measuring the size of the leading edge of transcripts at two different times of chase. Pairs of lanes were used to measure the distance traveled by the polymerase in the measured time. An average of the rates was determined from several pairs of lanes in at least three separate experiments.

While examining the effects of high salt, heparin, or Sarkosyl on elongation factor activity, we noticed a stimulation in the amount of RNA produced in treated reactions (Fig. 2; the lanes in which untreated extract was used were exposed twice as long as the other lanes). In preliminary experiments, we noticed that the magnitude of the stimulatory effect seemed to be inversely proportional to the length of the pulse time. To examine this effect more closely, we performed a time course of high-salt additions (Fig. 3) in which the pulse time was kept constant. Preinitiation complexes were formed during a 10-min preincubation of extract and template DNA. The complexes were then pulsed for 15 s under low-salt (60 mM) conditions and chased. The concentration of KCl was raised during the chase at times ranging from 0 to 2 min. At the end of the pulse, very few of the RNA molecules are longer than 50 nucleotides and so cannot be seen on the autoradiograph of the gel in Fig. 3 (0

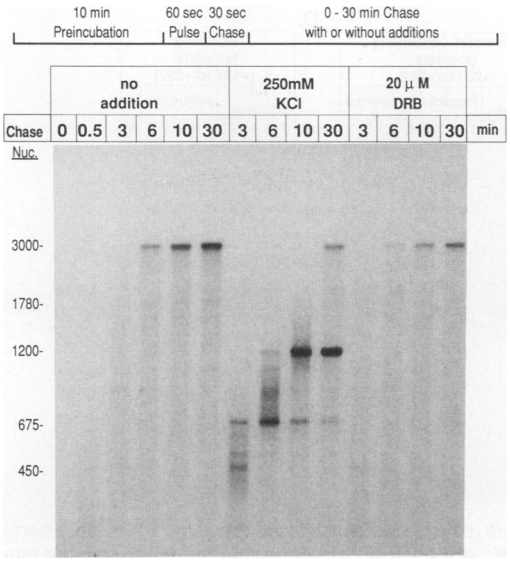

FIG. 4. Stability of elongation complexes containing RNA over 500 nucleotides in length. A pulse-chase protocol was followed, using K_c cell nuclear extract and A2(R/P) digested with *Eco*RI as described in Materials and Methods. Reactions were pulsed for 1 min (0-min time point with no addition). Addition of 250 mM KCl or DRB occurred after an initial chase of 30 s (0.5-min time point with no addition). Aliquots (12 μl) of a larger reaction mixture under various conditions were taken at increasing times and analyzed by denaturing 1.85% agarose gel electrophoresis as described in Materials and Methods. The sizes of DNA markers are given in nucleotides (Nuc.). The approximate lineup of these markers between two separate agarose gels is given.

chase lane). When salt is added immediately after the pulse, there is a dramatic stimulation of the amount of runoff RNA generated in 20 min of chase (compare results for the 20-min low-salt chase with those for the 20-min high-salt chase in Fig. 3). As the chase time that elapsed before the raising of the concentration of KCl was increased, the level of stimulation of runoff decreased. After 2 min of chase, very little stimulation occurred. Thus, it appears that there is a short time period after initiation during which raising the concentration of KCl causes the release of blocked RNA polymerase II complexes. Identical effects were found when Sarkosyl or heparin was substituted for 250 mM KCl (Fig. 2). Since initiation is inhibited by 250 mM KCl and only pulsed RNA is monitored, it is clear that increased initiation is not responsible for the increase in long transcripts (see also Fig. 7 in reference 40a).

Stability of elongation complexes containing transcripts over 500 nucleotides in length. Sarkosyl, heparin, or 250 mM KCl disrupts rapid transcriptional elongation, presumably by interfering with the activities of specific elongation factors. However, it is important to distinguish between the effects of heparin, Sarkosyl, or high salt on complexes already formed and the effects of the treatments on the formation of elongation complexes. To address this issue, we used a template containing the Act 5C promoter, which is capable of yielding runoff transcripts 3,000 nucleotides in length. The pulse-chase protocol used is diagrammed in Fig. 4. After pulse-labeling for 60 s, a 30-s chase was performed with high NTP levels to allow some elongation complexes to synthesize RNA transcripts between 500 and 1,000 nucleotides in length. Reaction conditions were then manipulated by the addition of chemical agents, and elongation was continued for the specified times. Examination of transcripts 1,000 to 3,000 nucleotides in length allows determination of the effects that different conditions have on the properties of elongation complexes that are far from the promoter and have had time to interact with other factors.

When untreated K_c cell nuclear extract is used in transcription reactions with the long template, significant numbers of transcripts resulting from paused elongation complexes are not detected (Fig. 4). A runoff transcript of 3,000 nucleotides can be detected in 3 min, consistent with a maximum elongation rate of about 1,000 nucleotides per min (Table 1). As was seen in Fig. 2 with shorter templates, the accumulation of long runoff required longer than expected considering the maximum elongation rate. This finding is due to a slow step in the generation of complexes capable of yielding long transcripts (40a). Adjusting the concentration of KCl to 250 mM after complexes have elongated greater than 500 nucleotides decreased the elongation rate and caused the appearance of short transcripts (Fig. 4). Many of these transcripts were chased into higher-molecular-weight species with time. This finding indicates that the observed transcripts are generated by pausing of the elongation complexes and not termination. Similar pausing is also seen when reaction mixtures are treated with 0.3% Sarkosyl or 1 mg of heparin per ml (data not shown).

The purine nucleoside analog DRB inhibits RNA polymerase II both in vivo (16, 64) and in vitro (11, 77). The most recent experimental evidence suggests that DRB inhibits transcriptional elongation in vitro by enhancing the pausing or premature termination of elongating polymerase molecules (11). To investigate the effects of DRB on elongating transcription complexes, we added 20 μM DRB to elongation complexes containing transcripts between 500 and 1,000 nucleotides in length. A time course of RNA synthesis indicates that DRB does not cause the appearance of transcripts due to paused complexes (Fig. 4). Additionally, the 3,000-nucleotide runoff transcript appeared at the same time as runoff using untreated extract, indicating that the elongation rate was unaffected (Fig. 4). However, at all time points DRB did have the effect of reducing the total amount of transcription complexes reaching the end of the template relative to reactions using untreated extract. This reduction is due to the inhibition of a factor, P-TEF (positive transcription elongation factor), which allows formation of elongation complexes capable of synthesizing long transcripts (40a).

DmS-II or *Drosophila* factor 5 does not stably interact with RNA polymerase II during elongation. If heparin, Sarkosyl, or 250 mM KCl inhibits the action of elongation factors, then pure RNA polymerase II should recognize similar pause sites during transcription of dC-tailed template containing the same sequence. To compare pause sites seen during promoter-driven transcription with the pause sites observed during transcription of the dC-tailed template, we have developed a system which allows the mapping of pause sites with single-nucleotide resolution. An oligonucleotide with a 5′ end corresponding to +1 of Act 5C was used as a primer in dideoxy sequencing reactions. Transcription reactions were performed by using the Act 5C promoter and nuclear extract with or without the addition of 1 mg of heparin per ml during the chase. Products from transcription reactions and DNA sequencing reactions were then electrophoresed on sequencing gels. The precise lineup between sequencing reactions and RNA transcripts was accomplished by comparing the dideoxycytosine sequencing lane with pausing of transcription complexes at cytosine residues during the

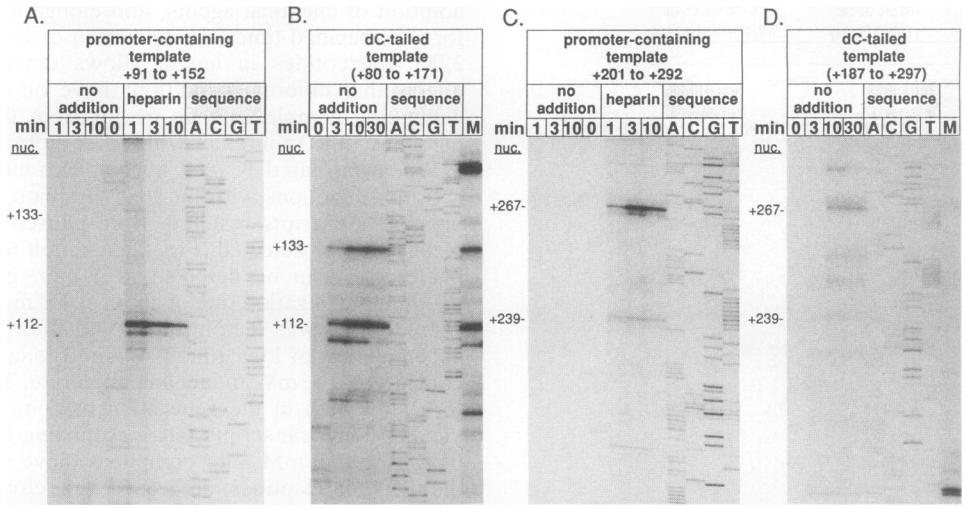

FIG. 5. Comparison of salt-, Sarkosyl-, or heparin-induced pause sites with pause sites recognized by pure RNA polymerase II. A pulse-chase protocol was used to perform transcription reactions, using either K_c cell nuclear extract on a promoter-containing template or purified *Drosophila* RNA polymerase II on a dC-tailed template. (A and C) The A2(R/P) template digested with *Pst*I was used for promoter-driven reactions. A 2-min pulse was followed by increasing chase times in the presence or absence of 1 mg of heparin per ml. Resulting transcripts were electrophoresed adjacent to the DNA sequence obtained by using a sequencing primer corresponding to +1 of Act 5C. A precise correlation between RNA transcripts and DNA sequence was obtained by comparing induced pausing during transcription under CTP-limiting pulse conditions (no addition, 0 min) with the dideoxycytosine DNA sequencing lane. (B and D) dC-tailed transcription reactions were carried out by using a pulse-chase protocol with purified RNA polymerase II and dC-A2 as described in Materials and Methods. Following a 3-min pulse, reactions were chased for increasing amounts of time. Products from transcription reactions were electrophoresed adjacent to the DNA sequence obtained by using a primer that correlates to the 5′ end of transcripts derived from the dC-tailed template. A precise lineup of RNA transcripts and DNA sequence was obtained by comparing the transcripts generated in reactions with limiting CTP (lane M) with dideoxycytosine sequencing lanes. In all panels, sequence numbering corresponds to positions relative to the RNA transcript obtained from the Act 5C promoter. The positions of several pause sites in common to both templates are indicated. Nuc., nucleotides.

CTP-limiting pulse (Fig. 5). To determine the sites of transcriptional pausing with use of pure RNA polymerase II and dC-tailed templates, a sequencing primer with the same 5′ end as transcripts derived from the dC-tailed template was used for sequencing reactions. RNA transcripts derived from transcription of the dC-tailed template by RNA polymerase II were electrophoresed along with corresponding sequencing reactions. A precise lineup between DNA sequence and the corresponding RNA transcripts was accomplished by comparing the dideoxycytosine sequencing lane with a transcription reaction performed at limiting CTP levels (Fig. 5). The pause sites encountered in the presence of heparin by RNA polymerase II initiated at the Act 5C promoter are also encountered by pure RNA polymerase II initiated on a dC-tailed template (Fig. 5). RNA polymerase II on the dC-tailed template paused at other sites as well. These additional sites could be caused by the lack of renaturation of the dC-tailed template during transcription, since *Drosophila* RNA polymerase II leaves its nascent transcript in heteroduplex with the template strand (70). This lack of renaturation during transcription of a dC-tailed template causes a reduction in the elongation rate of pure RNA polymerase II (70), consistent with an increase in the number of pause sites.

The results presented so far suggest that Sarkosyl, heparin, or 250 mM KCl interferes with efficient transcriptional elongation by inhibiting the activity of elongation factors. This was tested directly by using a dC-tailed template assay with purified RNA polymerase II, DmS-II, and factor 5. Because of differences in the properties of DmS-II and factor 5, different protocols were used to assay the activities of the two factors.

A 5-min, continuous-labeling protocol was used to assay factor 5 (see Materials and Methods). The ability of factor 5 to stimulate the elongation rate of RNA polymerase II is evident by the increased length of transcripts synthesized in the presence of the factor (Fig. 6). KCl (250 mM) or heparin (1 mg/ml) was added to the indicated reactions after elongation began. The stimulatory activity of factor 5 was almost eliminated by the addition of 250 mM KCl, and the addition of heparin to 1 mg/ml inhibited the activity of added factor 5 completely. The addition of salt has no effect on RNA polymerase II alone, but heparin at 1 mg/ml slightly inhibits the pure enzyme.

To determine how the activity of DmS-II is affected by heparin or 250 mM KCl, we used a pulse-chase protocol in which elongation was allowed to proceed for 10 min (see Materials and Methods). Under these conditions, 250 mM KCl has very little effect on transcription by RNA polymerase II except for a slight suppression of pausing at one site at about 300 nucleotides (Fig. 6). Heparin at 1 mg/ml causes a slight reduction in the elongation rate of RNA polymerase II. DmS-II suppressed pausing of RNA polymerase II at several sites and increased the size of the RNA synthesized during the 10 min (Fig. 6). The addition of KCl to 250 mM or the addition of heparin to 1 mg/ml reduced the ability of DmS-II to suppress pausing and stimulate the elongation rate.

Since elevated salt or heparin did not completely eliminate the activity of DmS-II, we analyzed the transcription products by scanning the autoradiograph depicted in Fig. 6 (Fig. 7). The scans confirm that both 250 mM KCl and heparin strongly inhibit the ability of DmS-II to suppress pausing, as evidenced by the increase in the intensities at 55 and 91 mm by 250 mM KCl and at 55, 61, and 91 mm by heparin. The

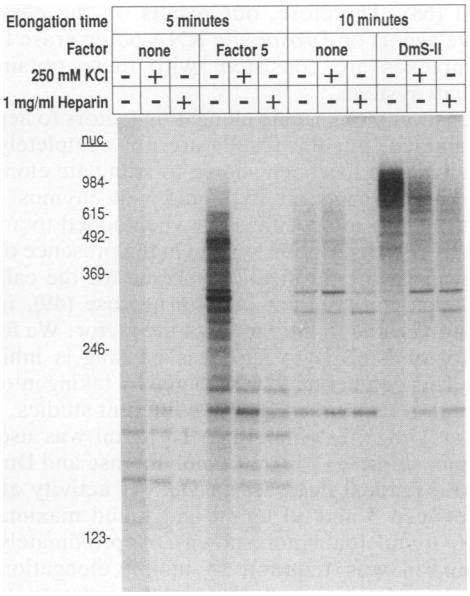

FIG. 6. Inhibition by salt and heparin of the activities of factor 5 and DmS-II. Reactions using dC-3500 and purified transcription components were performed as described in Materials and Methods. A 5-min continuous-labeling protocol was used for reactions on the left. Purified factor 5 (2 μl of Mono S fraction [47]) was added alone or in combination with 1 mg of heparin per ml or 250 mM KCl, as indicated. A pulse-chase protocol was used for reactions on the right. After a 2-min pulse, reactions were chased for 10 min. Pure DmS-II (4.5 μl of Mono S fraction 33 [69]) was added alone or in combination with 1 mg of heparin per ml or 250 mM KCl during the chase period. The sizes of DNA markers are given in nucleotides (nuc.).

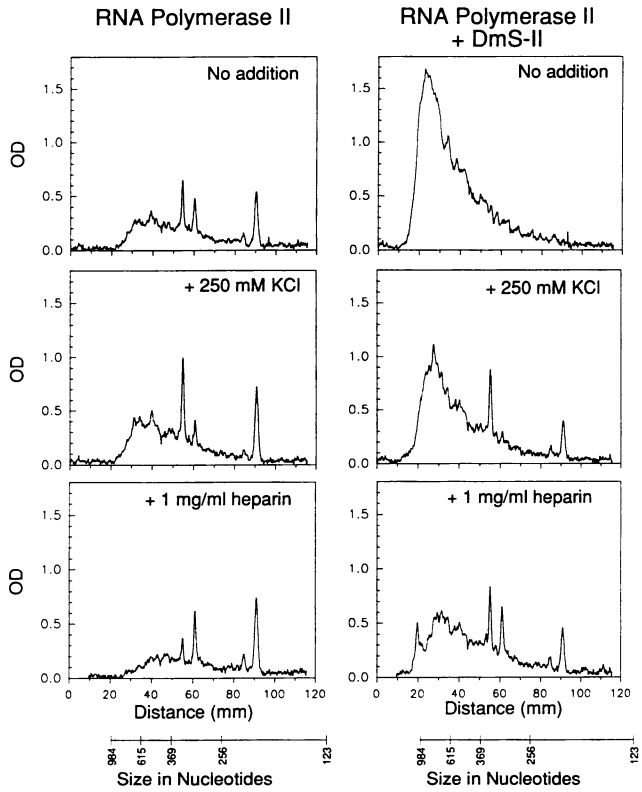

FIG. 7. Analysis of the effects of salt and heparin on the activity of DmS-II. The six right-hand lanes from the autoradiograph shown in Fig. 6 (10-min elongation time) were scanned with a Bio-Rad gel scanner, and the relative absorbance (optical density [OD]) was plotted against the distance from the well. The scans on the left show the effects of 250 mM KCl or heparin on pure RNA polymerase II, and the scans on the right show the effects of the reagents on RNA polymerase II in the presence of heparin.

stimulation of the maximum elongation rate during the 10-min chase was 2.1-fold for DmS-II alone and 1.8-fold for DmS-II in the presence of 250 mM KCl or heparin. The calculated size of the RNA at the position of the 95% point of the accumulated optical density from the scans was used to compute the elongation rates. Fold stimulations were the increase in the maximum elongation rate caused by DmS-II and took into account the 20% decrease in elongation rate of RNA polymerase II by heparin at 1 mg/ml. We have examined the effect of heparin on the transcription of RNA polymerase II and on the properties of DmS-II by performing titrations with heparin (data not shown). At levels of heparin at or below 300 μg/ml, there is no effect on the elongation rate of RNA polymerase II, but specific suppression of pausing by DmS-II is inhibited by concentrations of heparin greater than 10 μg/ml. At heparin concentrations that do not affect the elongation properties of RNA polymerase II, DmS-II is still able to increase the elongation rate, but not quite as much as in the absence of heparin. Therefore, heparin can eliminate the ability of DmS-II to suppress pausing at specific sites and can reduce but not eliminate the ability of DmS-II to increase the elongation rate. All of these results suggest that the suppression of specific pause sites and the increase in elongation rate by DmS-II may be at least partially separable activities of the factor.

We observed that the addition of DRB to transcription reaction mixtures with crude K_c cell nuclear extract had no apparent effect on RNA polymerase II elongation complexes containing RNA over 500 nucleotides in length (Fig. 4). We further investigated the effects of this chemical agent on transcription by using purified components. As outlined above, a continuous-labeling protocol was used to investigate the behavior of factor 5, and a pulse-chase protocol was used to examine the activity of DmS-II in the presence or absence of DRB at a final concentration of 20 μM. DRB has no effect on transcription by pure RNA polymerase II with either the continuous-labeling protocol or the pulse-chase protocol (Fig. 8). Further, the addition of DRB to 20 μM does not affect the ability of factor 5 or DmS-II to stimulate transcription in the dC-tailed template assay. The same concentration of DRB was found to completely inhibit the action of P-TEF, a factor which acts during the formation of elongation complexes which synthesize long RNA (40a).

DISCUSSION

An understanding of the requirements for efficient elongation is a prerequisite for studying transcriptional control through pausing or premature termination mechanisms. We show here that elongation complexes formed in *Drosophila* K_c cell nuclear extract after initiation at the Act 5C promoter are capable of efficient elongation over long distances and can maintain an elongation rate of 1,000 nucleotides per min. This high elongation rate is not maintained in the presence of 250 mM KCl, 1 mg of heparin per ml, or 0.3% Sarkosyl. Treated elongation complexes exhibited an elongation rate similar to that of pure RNA polymerase II. Using a dC-tailed

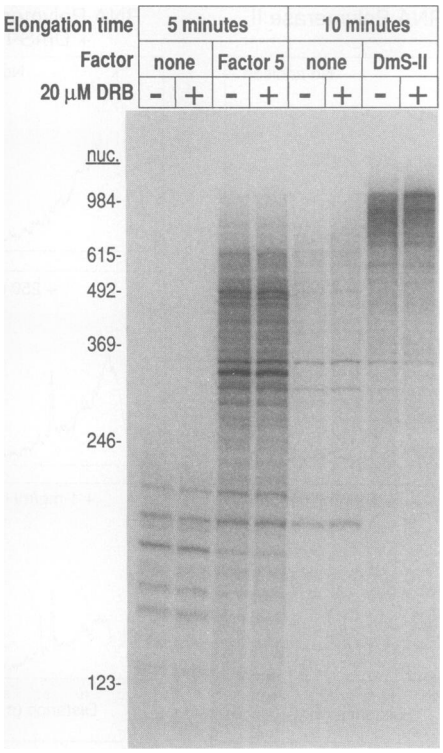

FIG. 8. Evidence of DRB does not inhibit RNA polymerase II, factor 5, or DmS-II. Reactions conditions were identical to those used for Fig. 6 except that DRB was added to a final concentration of 20 μM, as indicated. The sizes of DNA markers are given in nucleotides (nuc.).

template, we showed that the pause sites encountered by treated elongation complexes are a subset of those recognized by pure RNA polymerase II. Factor 5 and DmS-II are two factors found in K_c cell nuclear extract which affect elongation by pure RNA polymerase II, and we show here that they are both negatively affected by the disruptive agents in a completely defined system. We conclude these or other elongation factors are responsible for the efficient elongation seen in unfractionated K_c cell nuclear extract and that the disruptive agents eliminate the effects of these factors on transcription complexes initiated at a promoter.

The three disruptive agents have been used by others to study initiation and elongation. Sarkosyl has been useful in separating the initiation process into functional steps (18, 19, 24). Sarkosyl and heparin inhibit initiation and, if added after initiation, limit transcription to one round (13, 15, 18, 19, 24). Sarkosyl has been shown to enhance pausing by mammalian RNA polymerase II that has initiated from a promoter (2, 4, 18, 31, 54, 58, 75), presumably by interfering with the activity of elongation factors. Heparin has not been as widely used, but it has been found to increase a block to elongation by RNA polymerase II at a specific synthetic DNA sequence in vitro (2). Elevated levels of KCl have been found to affect elongation by mammalian RNA polymerase II when elongation complexes are chromatographed on Sephacryl S-1000. After treatment with 300 mM KCl, these complexes seem to be devoid of elongation factor activity (3). Mammalian S-II is not able to efficiently suppress pausing and subsequent termination at specific sites in the human histone H3.3 gene when elongation takes place in 150 mM KCl (68). Therefore, our results on the effect of the disruptive agents on *Drosophila* RNA polymerase II elongation complexes are consistent with those obtained with mammalian systems.

The sensitivity of specific elongation factors to heparin has been examined, but the results are not completely consistent. Human S-II has been shown to stimulate elongation in the presence of heparin (52), and calf thymus S-II can suppress pausing at specific sites when added to a reconstituted rat liver transcription system in the presence of heparin (54). However, the purification scheme for the calf thymus S-II includes elution from heparin agarose (49), indicating that heparin is able to interact with the factor. We found that the ability of DmS-II to suppress pausing is inhibited by heparin. This conflict may be resolved by taking into account the amount of heparin used in the different studies. We used heparin at 1 mg/ml, while 10 to 16 μg/ml was used in the mammalian studies. Using pure polymerase and DmS-II, we observed a gradual decrease in DmS-II activity at heparin levels between 3 and 30 μg/ml and found maximal effects above 30 μg/ml (data not shown). Approximately 10-fold more heparin was required to inhibit elongation factors present in unfractionated *Drosophila* extracts (data not shown). Presumably, it is necessary to add enough heparin to a reaction to bind all of the proteins present with affinity for heparin.

The three reagents which inhibit the action of elongation factors are known to disrupt protein-protein and protein-nucleic acid interactions. This inhibition rules out very tight association of the elongation factors with RNA polymerase II during elongation. Factor 5 and its mammalian counterpart TFIIF do bind to pure RNA polymerase II with high affinity (6, 47), but during elongation on a dC-tailed template, the affinity of factor 5 for *Drosophila* RNA polymerase II is much lower (47). Kinetic studies indicated that the factor only briefly interacts with elongating polymerase and is predominately free in solution (47). By interacting with the free factor, the disruptive agents might preclude further interactions with the elongation complex. DmS-II is not tightly associated with elongation complexes formed on dC-tailed templates either (69) and could be inhibited by the same mechanism. It is not clear whether the properties of elongation complexes on dC-tailed templates mirror those of elongation complexes formed after initiation at a promoter, but we show here that the effect of the three reagents used are similar in both assays, namely, elongation factors are inhibited. The use of disruptive agents does not allow us to say that elongation complexes formed after initiation from a promoter are devoid of stable interaction with elongation factors under normal elongation conditions because the reagents may be able to disrupt otherwise stable complexes. We have also found that dilution of the elongation complexes causes a reduction in elongation rate (data not shown), further suggesting that elongation factors are not stably associated with the complex.

One important conclusion reached from our results is that most polymerase molecules that initiate from a promoter are not able to produce long transcripts. We show here that this block to elongation can be relieved by adding Sarkosyl, heparin, or high salt during elongation. This early block to elongation complicates the study of elongation after initiation at a promoter. By using long templates and observing the properties of complexes containing long transcripts, we have attempted to separate the sensitivity of the formation of complexes from their stability. Our results indicate that the interpretation of studies which analyze only very short

transcripts may be very difficult. We have recently learned that a specific factor, P-TEF, is responsible for allowing some of the polymerase molecules to escape this early block in untreated extract (40a). This factor is limiting in nuclear extract, which causes a delay in the formation of complexes which can synthesize long transcripts. This slow step complicates the measurement of maximum elongation rates of untreated complexes and is responsible for the slow accumulation of long runoff transcripts. Another complication to the determination of elongation rates is that the concentration of other elongation factors, such as factor 5 and DmS-II, may be lower than that required to achieve the maximum rate. Slight variations in conditions might cause a dilution of factors which would reduce the elongation rate determined.

If elongation complexes in vivo are similar to elongation complexes in vitro, several mechanisms to control this phase of transcription are possible. The activity of elongation factors may be regulated simply by controlling the concentration of the factors. Increasing the concentration of elongation factors would increase the elongation rate or the ability to efficiently pass a specific pause site. The concentration or activity of the factors could be precisely regulated even after initiation to ensure synthesis of the proper level of mRNA. S-II is not required for initiation and therefore might allow independent control over the elongation phase of transcription. This could be especially important in very long genes such as the gene coding for dystrophin, which is over 2 million bp long and would require more than a day to produce a single mRNA molecule (43). The expression of genes related to S-II in specific cell types (25) suggests that some control over the elongation process might be exerted in a tissue-specific manner, by controlling the type of elongation factors that are present. Since factor 5 is required for initiation and also affects the elongation rate, the coordinate control of initiation and elongation might be regulated by the concentration or activity of this factor.

It has been suggested that DRB inhibits elongation by RNA polymerase II both in vivo (16, 64) and in vitro (11, 77). Our experiments clearly indicate the DRB does not affect elongation by transcription complexes containing RNA over 500 nucleotides in length that have initiated at a physiological promoter. Likewise, DRB does not affect transcription by purified RNA polymerase II initiated on a dC-tailed template and does not affect the activities of factor 5 or DmS-II. However, DRB does abolish the formation of elongation complexes that synthesize long transcripts (40a). This is evident in Fig. 4 in assays in which DRB is added to reactions after elongation has begun. The reduced amount of run-off RNA that is seen is due to inhibition of the formation of elongation complexes capable of synthesizing long transcripts. Complexes capable of synthesizing long transcripts that are already formed before the addition of DRB are not affected and exhibit a high elongation rate.

Now that we have determined some of the basic properties of elongation complexes formed after initiation at a promoter in *Drosophila* K_c cell nuclear extract, we can address more specific questions. It will be interesting to determine whether elongation complexes resulting from initiation at promoters that drive transcription of genes with widely different lengths have different properties. The details of the involvement of DmS-II in elongation can now be studied by using probes derived from the recent cloning of the gene for the factor (41). Most importantly, we are now in a position to begin to understand the biochemical details of the formation of elongation complexes and the specific factors involved.

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