Drosophila RNA Polymerase II Mutants That Affect Transcription Elongation*

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We have examined the properties of two Drosophila RNA polymerase II mutants, C4 and S1, during elongation, pyrophosphorolysis, and DmS-II-stimulated transcript cleavage. The C4 and S1 mutants contain a single amino acid substitution in the largest and second largest subunits, respectively. Compared with wild type, the C4 mutant had a lower elongation rate and was less efficient at reading through intrinsic elongation blocks. S1 had a higher elongation rate than wild type and was more efficient at reading through the same blocks. During elongation, C4 and wild type responded similarly to DmS-II and NH4+, whereas the S1 mutant was less responsive to both. Differences between the two mutants also appeared during DmS-II-mediated transcript cleavage and pyrophosphorolysis. During extended pyrophosphorolysis, S1 polymerase was fastest and C4 polymerase was slowest at generating the final pattern of transcripts. S1 and wild type were equal in the rate of extended DmS-II-mediated transcript cleavage, and C4 was slower. Our results suggest that the S1 mutation increases the time spent by the polymerase in elongation competent mode and that the C4 mutation may affect the movement of the polymerase.

Recent studies have revealed that the control of transcription elongation is an important target for the regulation of eukaryotic gene expression (1, 2). As the central component of the mRNA transcription machinery, RNA polymerase II itself is the final receptor of various kinds of interactions that activate or inactivate transcription elongation, and it carries out the fundamental catalysis of RNA chain elongation. However, functional roles played by its individual subunits during the process of elongation have not been thoroughly illuminated.

A number of mutations in RpII1215 and RpII140, the genes encoding the two large subunits of Drosophila RNA polymerase II, have been mapped at the DNA sequence level. Among them, the C4 mutation in RpII1215 changes amino acid 741 from Arg to His in the largest subunit (3), and the S1 mutation in RpII140 changes amino acid 728 from Ser to Cys in the second largest subunit (4). In vivo, the C4 mutation induces α-amanitin resistance and the Ubx effect (5, 6), whereas the S1 mutation suppresses the temperature-sensitive mutant phenotype caused by another mutation (WJ K1) in the largest subunit (7). Little is known about what functions of RNA polymerase II are altered and how transcription is affected by these and other mapped mutations, except that it was shown previously that the C4 mutant enzyme is resistant to α-amanitin and is slower in elongation in vitro (8, 9). Having previously mapped mutations in both RpII1215 and RpII140, we next initiated biochemical studies of a subset of the mutant enzymes that are amenable to purification (10). In our studies, we found that as for the C4 mutant enzyme, the S1 mutant enzyme is also different from wild type in elongation. Because the C4 mutation alters the largest subunit, whereas the S1 mutation alters the second largest subunit of Drosophila RNA polymerase II, the availability of C4 and S1 RNA polymerase II mutant enzymes provides an opportunity possibly to identify functional roles for the two large subunits during elongation and to dissect the molecular mechanism by which RNA polymerase II achieves efficient elongation.

Using cell-free transcription systems in which elongation complexes can be assembled, a number of factors have been identified that affect elongation properties of RNA polymerase II. For example, the Drosophila transcription factor, DmS-II, which was initially purified from Drosophila Kc cell nuclear extracts, has been shown to have a stimulatory effect on elongation in vitro (11, 12). DmS-II is a 36-kDa protein that is the Drosophila counterpart for mammalian S-II (TFIIS); the gene encoding DmS-II has been cloned and sequenced (13). DmS-II has been shown to reduce the time spent by RNA polymerase II at a subset of the numerous pause sites encountered on a dC-tailed template, but it does not stably bind to the elongation complex (11). DmS-II has also been shown to be necessary and sufficient to activate nascent transcript cleavage by Drosophila RNA polymerase II during transcription of a dC-tailed template, and the C-terminal half of DmS-II is required for its cleavage-activating function. A mechanism for pause suppression by DmS-II has been proposed. DmS-II binds to the paused polymerase, causes one cleavage event, and is then released from the complex. Elongation by the polymerase then allows a second encounter with the pause site and a second chance of passing the site. Complete pause suppression may require multiple transcript shortening events for some polymerase molecules (14).

In the study presented here, we investigate several elongation-related properties of wild type and mutant RNA polymerase II, namely recognition of intrinsic blocks to elongation, read-through in response to DmS-II, DmS-II-stimulated cleavage of nascent transcripts, and pyrophosphorolysis. We demonstrate that the C4 and S1 mutations affect different functional processes during elongation, and we discuss the implications of our observations.

EXPERIMENTAL PROCEDURES

Materials—Polymin P was purchased from ICN Biochemicals. Lyophilized ribonucleoside triphosphates were purchased from Pharmacia Biotech Inc. α-Amanitin, terminal deoxynucleotidyl transferase, and restriction enzymes were purchased from Boehringer Mannheim. 5′,6-

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**Drosophila RNA Polymerase II Mutants**

**Introduction**

The study of RNA polymerase II mutants in Drosophila has been instrumental in understanding the molecular mechanisms of transcription and RNA synthesis.

**Materials and Methods**

- **Embryo Buffer**: 25 mM HEPES (pH 7.6), 0.5 mM EDTA, 10% glycerol, 15 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 0.1% phenylmethylsulfonyl fluoride (PMSF). Solutions were filter-sterilized through one layer of Miracloth.
- **Polymin P**: A solution of Polymin P was stirred in droplets. After 20 min of stirring, the precipitate was washed in 50 ml of 0.1M HGKE using a glass-glass homogenizer.
- **C4 Enzyme**: The C4 enzyme's specific activity is approximately 40% lower than wild type and S1 enzymes. The resulting supernatant was collected after being filtered through one layer of Miracloth. Solid (NH₄)₂SO₄ (0.3 g/ml of polymin Peluate) was added (Sorvall). The resulting polymin Peluate was filtered through a 30-ml DE-52 column equilibrated in 0.12M HGAE and was centrifuged at 10,000 rpm (16,000 g) for 30 min in a T-865 ultracentrifuge rotor (Sorvall). The pellet was resuspended in 10–15 ml of HGE with a Dounce homogenizer to yield 30 min in a T-865 ultracentrifuge rotor (Sorvall). The pellet was resuspended in 10–15 ml of HGE with a Dounce homogenizer to yield ammonium sulfate enzyme, which was frozen in liquid nitrogen and stored at 5°C, and then digested by PstI and EcoRI and EcoHI fragments containing the actin 5C Drosophila promoter and initiation factors (19). The size distribution of radioactively labeled transcripts produced during a specific time period gives a measure of the elongation rate. Fig. 1A shows the RNA profiles when elongation assays were carried out with 100 mM KCl, Fig. 1B shows the RNA profiles when elongation assays were carried out with 50 mM KCl plus 80 mM (NH₄)₂SO₄, and Fig. 1C plots the maximum lengths of RNA produced by P2 or S1 at each time point.

**Results**

**1. Mutant RNA Polymerases II Show Abnormal Elongation Rates in Vivo**

To compare the elongation rates of mutant enzymes with wild type enzyme, we used a d-c-tailed template that allows the study of elongation in the absence of a physiological promoter and initiation factors (19). The size distribution of radioactively labeled transcripts produced during a specific time period gives a measure of the elongation rate. Fig. 1A shows the RNA profiles for P2 and S1 enzymes when elongation assays were carried out with 100 mM KCl, Fig. 1B shows the RNA profiles when elongation assays were carried out with 50 mM KCl plus 80 mM (NH₄)₂SO₄, and Fig. 1C plots the maximum lengths of RNA produced by P2 or S1 at each time point.
point under the two different salt conditions. We found that the S1 mutant enzyme was 2-fold faster in elongation than the P2 wild type enzyme when no NH₄⁺ was present. Under the same assay conditions, however, the C4 mutant enzyme was 50% slower in elongation (data not shown). In the presence of NH₄⁺, the elongation rate of P2 was increased by about 2-fold, but the elongation rate of S1 was almost not changed.

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Mutant RNA Polymerases II Show Abnormal Ability to Read through Intrinsic Elongation Blocks—During in vitro transcription from various dC-tailed templates, RNA polymerase II pauses or is blocked at numerous sites along the template, as revealed by discrete bands in the RNA profile. Reines et al. (17) characterized three sequence specific sites (TIa, TIb, and TII) in the first intron of the human histone H3.3 gene that efficiently block elongation by mammalian RNA polymerase II. Later, Christie et al. (20) showed that these three sites also block the elongation of purified yeast RNA polymerase II. Because C4 and S1 mutant enzymes showed abnormal elongation rates when transcribing a dC-tailed template (Fig. 1), we used dC-pGEMTerm, which contains TIa, TIb, and TII, to investigate if the two mutations would affect RNA polymerase II in recognizing and reading through the characterized intrinsic elongation blocks.

We found that both wild type and mutant Drosophila RNA polymerases II could be blocked at TIa, TIb, and TII in vitro. As shown in Fig. 2A, after a 5-min chase, besides the run-off transcripts (RO), transcripts with 3' ends at TIa, TIb, and TII are indicated by RO, TIa, TIb, and TII. Time points of the chase reaction are indicated at the top of each lane. B, quantitation was performed as described under “Experimental Procedures.” The ratios of RO/(RO + TIa) and (RO + TIa)/(RO + TIa + TIb) were calculated and plotted versus the time of chase. Symbols: filled circles, P2; open circles, S1; squares, C4.
Mutant RNA Polymerases II Act Differently in DmS-II–mediated Transcript Cleavage and Pyrophosphorolysis—Mammalian or yeast TFIIS has been shown to stimulate nascent transcript cleavage in elongation complexes stalled at the Tia site (20, 22). This cleavage precedes and is necessary for efficient read-through. In order to determine how the two mutations would affect RNA polymerase II in DmS-II–mediated transcript cleavage, we initiated transcription from dC-pGEMTerm and isolated elongation complexes stalled at Tia, Tlb, or TII using gel filtration spin columns. As shown in Fig. 5, for both wild type and mutant polymerases, when isolated elongation complexes were incubated with Dms-II, transcript shortening was observed and the patterns of cleaved transcripts were similar. When NTPs were added back to the elongation complexes that had been incubated with Dms-II for 60 min and had undergone extensive transcript cleavage, elongation of the shortened transcripts back to the Tia site was also observed (lanes 6, 12, and 18). These results indicate that the ternary elongation complex formed with either C4 or S1 mutant RNA polymerase II is still able to carry out Dms-II–mediated transcript cleavage and suggest that the shortened transcripts remain stably associated with the complex. Quantitation of the remaining transcripts with 3′ ends at Tia revealed that after 10 min, 90% of the Tia transcripts in the P2 or C4 containing elongation complexes were cleaved, whereas only 57% were cleaved in the S1-containing complexes, consistent with S1 being different in interacting with Dms-II. In order to look in more detail at Dms-II–mediated transcript

Tia) and (RO + Tia)/(RO + Tia + Tlb) were different for wild type and mutant enzymes. As compared in Fig. 2B, after 5 min of chase, the ratios of RO/(RO + Tia) were 34, 11, and 54% for P2, C4, and S1, respectively; after 60 min of chase, the ratios increased to 58, 49, and 62%, respectively. After 5 min of chase, the ratios of (RO + Tia)/(RO + Tia + Tlb) were 64, 53, and 71% for P2, C4, and S1, respectively; after 60 min of chase, the ratios increased to 82, 78, and 82%, respectively. In these experiments, the ratios of RO/(RO + Tia) and (RO + Tia)/(RO + Tia + Tlb) reflect the half-life of pausing at Tia and Tlb, respectively, but they are also influenced by the timing of the arrival of polymerases from other earlier pause sites and by the number of polymerases that become arrested (unable to proceed) at Tia and Tlb. The different ratios clearly indicate a functional difference between the polymerases. Compared with P2 wild type, the C4 mutant enzyme reads less efficiently through Tia and Tlb, whereas the S1 mutant enzyme reads more efficiently through the same blocks.

Mutant RNA Polymerases II Show Different Response to Dms-II—Mammalian TFIIS or the yeast TFIIS analog, P37, has been shown to stimulate mammalian or yeast RNA polymerase II to read through Tia, Tlb, and TII (20, 21). Because C4 and S1 mutants were different from wild type in reading through Tia and Tlb when no elongation factors were present, we further investigated how they would respond to Dms-II. We found that Dms-II was able to promote Drosophila RNA polymerase II to read through intrinsic elongation blocks, but although P2 and C4 were stimulated by Dms-II to a similar degree, S1 was much less responsive to the action of Dms-II.

A time course of elongation is shown in Fig. 3A and the quantitation is shown in Fig. 3B. After 5 min of chase in the presence of 2.2 nM Dms-II (note that before Dms-II was added there was a 5-min initial chase in the absence of Dms-II), the ratio of RO/(RO + Tia) for P2 was 79%, increasing from 45% after 10 min of chase in the absence of Dms-II (Fig. 2A, 10-min point); this represents a 1.8-fold stimulation. Within the period of 60 min, the presence of Dms-II resulted in an average of 1.7-fold increase in read-through of Tia by P2 (n-min points in Fig. 3 versus n + 5-min points in Fig. 2). Similarly, Dms-II resulted in an average of 1.7-fold increase in read-through of Tia by C4, although compared with P2, the C4 mutant remained less efficient at reading through Tia. In contrast, the same amount of Dms-II only resulted in an average of 1.2-fold increase in read-through of Tia by S1 mutant enzyme (compare Figs. 3 and 2).

The experiment shown in Fig. 4 also examines the effect of Dms-II by comparing the RNA profile generated in its absence or presence. For P2 wild type, although the maximum elongation rate (determined from the leading edge of the transcript distribution at each time point) was not stimulated very much, the quantity of pulse-labeled transcripts was greater in the presence of Dms-II, indicating that Dms-II increased the number of P2 polymerase molecules that passed through early pause sites (compare lanes 5, 6, 7, and 8 with lanes 1, 2, 3, and 4). Although there was generally much more labeled RNA after an 8-min chase in the presence of Dms-II than in the absence of Dms-II, an RNA of about 140 nucleotides (Fig. 4, indicated by the arrow) diminished when Dms-II was present (compare lane 8 with lane4), indicating pausing of P2 at this specific site was reduced by Dms-II. However, for the S1 mutant, there was not much increase in the amount of labeled RNA when Dms-II was present (compare lanes 13, 14, 15, and 16 with lanes 9, 10, 11, and 12), and there was not much decrease in the 140-nucleotide RNA (compare lane 16 with lane 12), confirming that S1 was less responsive to the action of Dms-II.

Mutant RNA Polymerases II Act Differently in DmS-II–mediated Transcript Cleavage and Pyrophosphorolysis—Mammalian or yeast TFIIS has been shown to stimulate nascent transcript cleavage in elongation complexes stalled at the Tia site (20, 22). This cleavage precedes and is necessary for efficient read-through. In order to determine how the two mutations would affect RNA polymerase II in DmS-II–mediated transcript cleavage, we initiated transcription from dC-pGEMTerm and isolated elongation complexes stalled at Tia, Tlb, or TII using gel filtration spin columns. As shown in Fig. 5, for both wild type and mutant polymerases, when isolated elongation complexes were incubated with Dms-II, transcript shortening was observed and the patterns of cleaved transcripts were similar. When NTPs were added back to the elongation complexes that had been incubated with Dms-II for 60 min and had undergone extensive transcript cleavage, elongation of the shortened transcripts back to the Tia site was also observed (lanes 6, 12, and 18). These results indicate that the ternary elongation complex formed with either C4 or S1 mutant RNA polymerase II is still able to carry out Dms-II–mediated transcript cleavage and suggest that the shortened transcripts remain stably associated with the complex. Quantitation of the remaining transcripts with 3′ ends at Tia revealed that after 10 min, 90% of the Tia transcripts in the P2 or C4 containing elongation complexes were cleaved, whereas only 57% were cleaved in the S1-containing complexes, consistent with S1 being different in interacting with Dms-II. In order to look in more detail at Dms-II–mediated transcript cleavage and Pyrophosphorolysis—Mammalian or yeast TFIIS has been shown to stimulate nascent transcript cleavage in elongation complexes stalled at the Tia site (20, 22). This cleavage precedes and is necessary for efficient read-through. In order to determine how the two mutations would affect RNA polymerase II in DmS-II–mediated transcript cleavage, we initiated transcription from dC-pGEMTerm and isolated elongation complexes stalled at Tia, Tlb, or TII using gel filtration spin columns. As shown in Fig. 5, for both wild type and mutant polymerases, when isolated elongation complexes were incubated with Dms-II, transcript shortening was observed and the patterns of cleaved transcripts were similar. When NTPs were added back to the elongation complexes that had been incubated with Dms-II for 60 min and had undergone extensive transcript cleavage, elongation of the shortened transcripts back to the Tia site was also observed (lanes 6, 12, and 18). These results indicate that the ternary elongation complex formed with either C4 or S1 mutant RNA polymerase II is still able to carry out Dms-II–mediated transcript cleavage and suggest that the shortened transcripts remain stably associated with the complex. Quantitation of the remaining transcripts with 3′ ends at Tia revealed that after 10 min, 90% of the Tia transcripts in the P2 or C4 containing elongation complexes were cleaved, whereas only 57% were cleaved in the S1-containing complexes, consistent with S1 being different in interacting with Dms-II.
cleavage and compare it with pyrophosphorylation, we isolated elongation complexes stalled at a major elongation block after the incorporation of 13 nucleotides with immobilized dC-3025 urea-TBE gel. Lengths of the DNA size standards (M) are given in nucleotides, time points of the chase reaction are indicated at the top of each lane. The 140-nucleotide RNA is indicated by an arrow.

**Fig. 5.** Comparison of DmS-II-stimulated transcript cleavage from T1a by P2 wild type, C4 mutant, and S1 mutant RNA polymerase II. Elongation complexes were formed on dC-pGEMTerm from TIa by 4.5 nM DmS-II. Purified RNAs were analyzed on a 5% polyacrylamide-6 M urea-TBE gel. The run-off transcripts and transcripts with 3' ends at T1a, T1b, and TII are indicated by RO, T1a, T1b, and TII. The major cleavage products are indicated by the arrows.

**DISCUSSION**

We have examined the properties of two Drosophila RNA polymerase II mutants in elongation, recognition of intrinsic elongation blocks, read-through in response to DmS-II, transcript cleavage in response to DmS-II, and pyrophosphorolysis. Our results show that the two mutations affect distinct functions associated with the two large subunits of RNA polymerase II. The C4 mutation in the largest subunit probably affects the translocation of the polymerase such that the forward and backward movement of the mutant elongation complex is slowed down. The S1 mutation in the second largest subunit probably affects the conformation of the polymerase and increases the time spent by the polymerase in the elongation competent mode such that the mutant enzyme is less responsive to the action of DmS-II and NH₄⁺.

It has been shown previously that the C4 mutant polymerase is resistant to α-amanitin in RNA polymerization due to decreased binding affinity to the toxin (8). We found that in the presence of 1 μg/ml of α-amanitin, the DmS-II-stimulated cleavage of nascent transcripts by wild type polymerase was not observed within 60 min, confirming the inhibitory effect of α-amanitin on this process (14, 23–25). In contrast, the cleavage by C4 mutant was still observed (data not shown), consistent with C4 not binding α-amanitin normally. Although the detailed inhibitory mechanism of α-amanitin is yet to be elucidated, recent studies suggest that α-amanitin inhibits pyrophosphorolysis and DmS-II-mediated transcript cleavage differently. For example, the toxin allows (slowed) pyrophosphorolysis within a paused elongation complex but completely blocks DmS-II action (26).

Because the response of C4 to DmS-II and NH₄⁺ parallels that of the wild type, the structural features involved in interacting with DmS-II and NH₄⁺ are probably not affected by the C4 mutation. Furthermore, because C4 carries out initial DmS-II-mediated transcript cleavage and pyrophosphorolysis as well as the wild type enzyme, the catalytic steps during these two processes may not be affected, either. It is possible that although the C4 mutation reduces the affinity of the enzyme for α-amanitin, it also introduces certain functional changes similar to those that would be caused by α-amanitin binding. One possibility is that the forward and backward translocation is slowed. This possibility is consistent with our observations that C4 is slower in reading through elongation blocks and is also slower in extended transcript shortening.

Three regions of the largest subunit of RNA polymerase II have been implicated previously in binding of TFIIIS. Sawadogo et al. (27) analyzed the interaction of yeast TFIIIS with RNA-
plexes were incubated for the indicated time and then washed at this step. Elongation complexes were formed on immobilized DmS-II as described under ‘Experimental Procedures’ and as indicated by the diagram in the lower portion of this figure. The black circle with W symbol indicates that the elongation complexes were washed at this step. Elongation complexes were incubated for the indicated times with 1 mM pyrophosphate (A) or 1 mM DmS-II (B). RNAs were analyzed on an 18% polyacrylamide-6 M urea-TBE gel. Transcripts sizes are indicated in nucleotides.

FIG. 6. Comparison of pyrophosphorolysis and DmS-II-mediated transcript cleavage from the 13-mer by P2 wild type, C4 mutant, and S1 mutant RNA polymerase II. Elongation complexes were formed on immobilized Dc-3025 as described under “Experimental Procedures” and as indicated by the diagram in the lower portion of this figure. The black circle with W symbol indicates that the elongation complexes were washed at this step. Elongation complexes were incubated for the indicated times with 1 mM pyrophosphate (A) or 1 mM DmS-II (B). RNAs were analyzed on an 18% polyacrylamide-6 M urea-TBE gel. Transcripts sizes are indicated in nucleotides.

- **Pol II** +DNA
- **GTP**
- **ATP**
- **CTP**
- **NTPs**
- **DmS-II**
- **PPI**
- **or**
- **Stop**

### A. 1 mM PPI

| P2 | S1 | C4 |
|----|----|----|
| min. | 0 | 3 | 10 | 30 | 90 |
| RO- | 0 | 3 | 10 | 30 | 90 |

### B. 1 mM DmS-II

| P2 | S1 | C4 |
|----|----|----|
| min. | 0 | 1 | .3 | 1 | 3 | 10 |

**Pol II**

- **GTP**
- **ATP**
- **CTP**
- **NTPs**
- **DmS-II**
- **PPI**
- **or**
- **Stop**

**Indicated Time**

10' 0.5' 8'
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