Drosophila Factor 2, an RNA Polymerase II Transcript Release Factor, Has DNA-dependent ATPase Activity*

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Control of transcription elongation has been recognized as an important mechanism regulating eucaryotic gene expression (1–3). The strategy to achieve the elongation control is to first apply a negative elongation potential to block and ultimately terminate RNA polymerase II transcription. The premature termination is then regulated by positive factors which enable RNA polymerase II to read through the block to transcription elongation. Based on our in vitro studies of Drosophila RNA polymerase II transcription, we proposed a model for the control of elongation by RNA polymerase II that incorporated the function of both negative and positive factors (4, 5). According to the model, all RNA polymerase II molecules that initiate from a promoter are destined to produce only short transcripts because of the action of negative transcription elongation factor (N-TEF). Escape from this abortive elongation into productive elongation requires the action of positive transcription elongation factor (P-TEF). P-TEFb, one of the components of P-TEF, has been purified and was found to phosphorylate the C-terminal domain of the largest subunit of RNA polymerase II (6, 7). Factor 2, the first identified component of N-TEF, has been purified to apparent homogeneity as a monomer with a molecular mass of 154 kDa (8). Factor 2 associates with early elongation complexes and causes premature termination by releasing RNA polymerase II transcripts generated from early elongation complexes in an ATP-dependent manner (8).

ATP has been found to be required for factor-dependent termination in several transcription systems. Autoantigen La, an RNA polymerase III transcription termination factor, hydrolyzes ATP to provide energy for the termination reaction (9). The ATPase activity of La was dependent on RNA binding and was only activated by double-stranded RNA or DNA-RNA hybrid (9). In vaccinia virus, transcription termination mediated by virus-encoded termination factor (VTF) also requires ATP hydrolysis, though VTF itself is not directly responsible for the ATP hydrolysis. Instead, VTF is required to recognize a special sequence in the nascent RNA and transduce the signal to an activity named factor X, which catalyzed the ATP hydrolysis required for transcript release (10). Rhino-dependent transcription termination in Escherichia coli requires ATP hydrolysis catalyzed by rho, an RNA-dependent ATPase (11–13). Rho binds to RNA and translocates along nascent transcripts to track elongating complexes and catalyzes transcript release by hydrolyzing ATP. Though ATP is required for the transcript release activity of factor 2, the role of the nucleotide and its functional relationship with factor 2 is not clear.

To understand the mechanism of factor 2 function, in this study, we investigated the importance of ATP hydrolysis in the transcript release reaction and tested for the potential ATPase activity of factor 2. In addition, we examined the transcript release activity of factor 2 using a defined system based on an immobilized dC-tailed DNA template and pure RNA polymerase II.

EXPERIMENTAL PROCEDURES

Transcript Release Assays—The transcript release assay using an immobilized Drosophila actin 5C template was described (5, 8). Transcript release assays using an immobilized Drosophila actin 5C template utilized the same protocol, except that the generation of elongation complexes was as described (14). RNase H (15) was added to dC-tailed template assays to cause template renaturation as described in the text. To examine the sensitivity of RNA transcripts to RNase A or RNase H, isolated elongation complexes were incubated with the indicated nucleases for 15 min at 25 °C and then stopped with 18 μl of HKE (20 mM HEPES, pH 7.6, 60 mM KCl, and 10 mM EDTA). To test the effect of α-amanitin on the transcript release activity of factor 2, high salt-washed early elongation complexes were first incubated with 2 μg/ml α-amanitin at room temperature for 2 min before factor 2 and ATP or dATP were added to the reaction mixture. Labeled transcripts were quantitated using a Packard InstantImager. Assays used factor 2 (0.7 μM) from glycerol gradient fraction 15 described earlier (8).

ATPase Assay—The ATPase activity of Drosophila factor 2 was assayed by measuring the release of inorganic phosphate (P_i) from [γ-32P]ATP (Amersham Life Science, Inc.). The ATPase assay (8 μl) contained 20 mM HEPES (pH 7.6), 5 mM MgCl₂, 0.2 mg/ml bovine serum albumin, 2 mM dithiothreitol, 1 μCi of [γ-32P]ATP, 5 μM ATP, with indicated amount of factor 2 and double-stranded Act5C (4) DNA template linearized with HpaI. The reactions were initiated by the addition of 2 μl of 4 × label mixture (containing 80 mM HEPES, 20 mM MgCl₂,

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¶ The abbreviations used are: N-TEF, negative transcription elongation factor; P-TEF, positive transcription elongation factor; ATP5, adenosine 5‘-O-(thiotriphosphate) (ATPγS), adenosine 5‘-(β,γ-imino)triphosphate (AMP-PNP), or other NTPs do not support the activity. Factor 2 demonstrated a strong DNA-dependent ATPase activity that correlated with its transcript release activity. At 20 μg/ml DNA, the ATPase activity of factor 2 had an apparent Km (ATP) of 28 μM and an estimated Kcat of 140 min⁻¹. Factor 2 caused the release of nascent transcripts associated with elongation complexes generated by RNA polymerase II on a dC-tailed template. Therefore, no other protein cofactors are required for the transcript release activity of factor 2. Using the dC-tailed template assay, it was found that renaturation of the template was required for factor 2 function.

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8 mM dithiothreitol, 1 μCi of [γ-32P]ATP, 20 μM ATP) and incubated for 20 min at 25 °C. The reactions were terminated by the addition of 1 μl of 0.5 M EDTA and placed on ice. The reaction mixture (1 μl) was spotted on polyethyleneimine (PEI) thin layer chromatography plate (E. Merck). The thin layer chromatography plate was developed by using 1 mM formic acid and 0.5 M lithium chloride as the running buffer. The percentage of the ATP hydrolyzation was determined by quantitating the amount of the released [32P]P, and the unhydrolyzed [γ-32P]ATP using a Packard InstantImager.

To determine the Km for the ATPase activity of factor 2, initial rates are measured at different concentrations of substrate ranging from 5 to 600 μM ATP. The ATPase assays to measure the initial rates were carried out in the presence of 20 μg/ml of HpaI linearized actin5C transcription template (4). The initial rates (pmol of ATP/min) versus the substrate ATP concentration (μM) were plotted, and the apparent Km value was obtained from the double reciprocal plot of 1/V versus 1/[ATP].

RESULTS

Only ATP and dATP Support Transcript Release Activity of Factor 2—Previous work showed that ATP was required for Drosophila factor 2 to cause release of transcripts by RNA polymerase II (8). Here we examined the nucleotide specificity for the transcript release activity. Using an immobilized DNA fragment containing the Act5C promoter, early elongation complexes were generated and washed with 1 mM KCl solution to remove the associated factor 2 (Fig. 1, diagram at top). The high salt-washed complexes were incubated with the indicated nucleotide in the presence or absence of purified factor 2 for 5 min. Released transcripts were separated from the template-associated transcripts by magnetic concentration. Only ATP and dATP supported the transcript release activity, while GTP, CTP, UTP, dGTP, dCTP, or dTTP did not (Fig. 1A). The nonhydrolyzable ATP analogs ATPγS or AMPPNP did not support the transcript release activity (Fig. 1A), suggesting that the hydrolysis of ATP was required. α-Amanitin, which binds to RNA polymerase II and inhibits transcription elongation (16), was also examined for its effect on the activity of factor 2. The isolated elongation complexes were first incubated with α-amanitin and then treated with factor 2 and the indicated nucleotide (Fig. 1A). α-Amanitin did not affect the activity of factor 2 to release RNA transcripts in the presence of either ATP or dATP (Fig. 1A). Though both ATP and dATP can be utilized by factor 2 to release RNA transcripts, 10 μM ATP was utilized with the same efficiency of 300 μM dATP (Fig. 1B). It is not likely that the activity seen with dATP is due to contamination with ATP since the dATP contains less than 0.5% ATP (information provided by Pharmacia Biotech).

Purified Factor 2 Has a DNA-dependent ATPase Activity—Since ATP hydrolysis was required for the function of factor 2, we determined if factor 2 had an ATPase activity. A standard thin layer chromatography assay that separated free [32P]phosphate from ATP was performed, and factor 2 alone was found to have a low level of ATPase activity (Fig. 2, A and B). Previous experiments indicated that factor 2 associated with early elongation complexes (8) and had a strong affinity to DNA cellu-
quired a divalent cation with Mg\(^{2+}\) and concentration of DNA was increased up to 80 m\(^{g}\)ml, increasing factor 2 caused a linear increase in ATPase activity until the percent hydrolysis was so great that ATP became limiting during the reaction (Fig. 2, A). Although our previous results suggested that factor 2 could function alone with the nascent transcripts were released (Fig. 5, A). These results suggested that factor 2 could work alone with the small amount of transcripts sensitive to RNase A or resistant to RNase H degraded 80% of the transcripts associated with elongation complexes (beads) but did not affect the small amount of transcripts generated by RNA polymerase II. It has been found that most of the transcripts generated by dC-tailed templates. It has been found that most of the transcripts were resistant to factor 2.

Although it was possible that a protein cofactor might be required to achieve complete release of transcripts in the dC-tailed template assay, it was more likely that the factor 2-resistant complexes resulted from an unusual feature of transcription of dC-tailed templates. It has been found that most of the transcripts generated by Drosophila RNA polymerase II are in heteroduplex with the template strand (RNase H sensitive), which prevents the renaturation of the template (15). As expected, RNase H degraded 80% of the transcripts associated with elongation complexes (beads) but did not affect the small amount of transcripts found in the supernatant fraction (Fig. 5B). Consistent with this result, less than 20% of the transcripts associated with elongation complexes were sensitive to RNase A, whereas the transcripts in the supernatant were completely sensitive (Fig. 5B). The fraction of transcripts released from elongation complexes by factor 2 was similar to the fraction of transcripts sensitive to RNase A or resistant to

# DNA-dependent ATPase of Transcript Release Factor

**FIG. 3.** Correlation of the transcript release activity of factor 2 with ATPase activity. A, ATPase assay (with 20 m\(^{g}\)ml DNA) across the glycerol gradient sedimentation fractions generated in the final purification step of factor 2. B, graphic representation of ATPase and transcript release activity. ATPase activity in panel A was quantitated using a Packard InstantImager, and transcript release was quantitated with a Bio-Rad model GS-670 imaging densitometer and the autoradiograph of the experiment shown in Fig. 2F of Ref. 8. The released RNA (%) was calculated by comparing the amount of released transcripts with the total transcripts in the reaction.

**TABLE I**

| ATP | ATP hydrolyzed |
|-----|---------------|
| mM  | pmol/min      |
| 3   | 0.60          |
| 5   | 0.93          |
| 10  | 1.62          |
| 30  | 3.02          |
| 100 | 4.71          |
| 300 | 6.40          |
| 600 | 7.12          |

The ATPase activity of factor 2 was assayed at indicated ATP concentrations in the presence of 20 m\(^{g}\)ml double-stranded DNA. The ATPase activity of factor 2 was determined by measuring the initial velocity of ATP hydrolysis at different concentrations of ATP (Table I). A double reciprocal plot (1/V versus 1/[ATP]) of the data (Fig. 4) yielded an apparent $K_{m(ATP)}$ for factor 2 of 28 \(\mu\)M and a $V_{max}$ of 6.25 pmol of ATP hydrolyzed/min. After estimating the amount of factor 2 based on the absorbance at 280 m\(^{g}\)ml, the turnover number $K_{cat}$ was calculated to be about 140 min\(^{-1}\). These results indicated that factor 2 is very active in hydrolyzing ATP under conditions normally used for transcription.

**Factor 2 Causes Transcript Release from Elongation Complexes Formed on a dC-tailed Template**—To determine if factor 2 was competent to function as a transcript release factor without additional protein cofactors, we used a dC-tailed template that allows efficient transcription initiation by pure RNA polymerase II. As diagrammed in (Fig. 5, top), initiation of prebound RNA polymerase II was accomplished with a 30-s pulse. The labeled complexes were isolated and then chased to generate transcripts between 50 and 250 nt in length. The resulting elongation complexes containing transcripts longer than 20 nt were examined for their sensitivity to factor 2 (transcripts less than 20 nt were analyzed separately and will be discussed below). When the isolated elongation complexes were incubated with only buffer or ATP, less than 10% of the transcripts were released (Fig. 5A). When the reactions were supplemented with factor 2 and ATP, an additional 17% of the nascent transcripts were released (Fig. 5A). The transcripts resistant to release by factor 2 did not result from the use of a subsaturating level of factor 2 since increasing the amount of factor 2 by 3-fold did not cause further release (Fig. 5A). These results suggested that factor 2 could function alone with the caveat that most of the transcripts were resistant to factor 2.

To provide further evidence that the DNA-dependent ATPase was associated with factor 2, an ATPase assay was performed across the glycerol gradient fractions of the final step of factor 2 purification (8). Results from both an ATPase assay and a transcript release assay across the same set of fractions (data from Ref. 8) were quantitated and compared (Fig. 3). The ATPase correlated with the transcript release, suggesting that the DNA-dependent ATPase activity was intrinsic to factor 2.

Primary kinetic parameters for the ATPase activity of factor 2 were also determined under conditions similar to that used for transcription except that only ATP was used. The apparent $K_{m(ATP)}$ for factor 2 was determined by measuring the initial velocity of ATP hydrolysis at different concentrations of ATP (Table I). A double reciprocal plot (1/V versus 1/[ATP]) of the data (Fig. 4) yielded an apparent $K_{m(ATP)}$ for factor 2 of 28 \(\mu\)M and a $V_{max}$ of 6.25 pmol of ATP hydrolyzed/min. After estimating the amount of factor 2 based on the absorbance at 280 m\(^{g}\)ml, the turnover number $K_{cat}$ was calculated to be about 140 min\(^{-1}\). These results indicated that factor 2 is very active in hydrolyzing ATP under conditions normally used for transcription.

2 Z. Xie and D. Price, unpublished results.
RNase H. This suggested that factor 2 caused release of only transcripts that were not in heteroduplex. Indeed, the transcripts found in factor 2-resistant complexes were sensitive to RNase H (last two lanes in Fig. 5B). The transcripts around 13 nt in length were resistant to factor 2 function (Fig. 5A) and were also resistant to RNase H and RNase A digestion (Fig. 5B).

In a complementary experiment, elongation complexes generated in the presence of RNase H were examined for sensitivity to factor 2. To confirm that the transcripts were no longer in heteroduplex, the complexes were incubated with either RNase H or RNase A. The transcripts present in these elongation complexes were not sensitive to RNase H but were degraded by RNase A (Fig. 6A). When these elongation complexes were incubated with only ATP, a low level (about 15%) of transcripts was released in the supernatant fraction, which is similar to the level of transcript release during the RNase H incubation (Fig. 6B). When factor 2 was added along with ATP, transcript release increased to 35% (Fig. 6B). When the level of factor 2 was tripled, transcript release was increased to a total of more than 80% (Fig. 6B). Transcript release was dependent on ATP as found before (data not shown). These results indicate that no other protein factors were required for the transcript release activity of factor 2 and that only transcripts displaced from the template strand were released by factor 2.

**DISCUSSION**

Factor 2 has been shown to promote the dissociation of RNA polymerase II transcripts from ternary elongation complexes in an ATP-dependent manner (8). To understand how factor 2 causes transcript release and ultimately elucidate its role in elongation control, we investigated the biochemical properties of the factor in defined *in vitro* systems. With an immobilized template assay, we found that hydrolysis of ATP was required for the transcript release activity of factor 2 and that only dATP could substitute for ATP. Using pure factor 2, we found that it exhibited an ATPase activity that was strongly dependent on DNA. We employed a dC-tailed template assay with pure RNA polymerase II and found that factor 2 did not require any other protein cofactors. In addition, we demonstrated that transcript release occurred only under conditions that caused transcript displacement and template renaturation.

Even though factor 2 shares a requirement for ATP hydrolysis with other termination factors, our results suggest that factor 2 utilizes a different mechanism for transcript release. ATP hydrolysis is required for termination by vaccinia VTF (17, 18), *E. coli* rho (11–13), and RNA polymerase III termina-
Nucleic acids have been shown to play an important role in activating various ATPases. RNA is an essential cofactor for the ATPase rho. RNA stabilizes rho as a monomer of 154 kDa with little ATPase activity in the absence of DNA. It is possible that DNA induces a conformational change or causes oligomerization of factor 2 and, thereby, stimulates its ATPase activity.

The characterization of factor 2 function using a dC-tailed template assay revealed that transcript release was blocked when the RNA remained in heteroduplex with the template. It is possible that extensive heteroduplex formation stabilizes the association of the RNA with the template and thus increases the energy required to cause transcript release beyond that provided by factor 2. However, another more interesting explanation is that double-stranded DNA, not RNA/DNA hybrid, is required upstream of elongation complexes for factor 2-mediated transcript release. This possibility is suggested by the finding that the 13-mer generated on a dC-tailed template was resistant to the transcript release activity of factor 2 (Fig. 5A). Factor 2 has been shown to dissociate transcripts as short as 9 or 10 nucleotides in length generated from promoter containing templates (8). It is possible that the resistance of 13-mer to factor 2 is due to the lack of double-stranded DNA upstream of the ternary complex. However, previous studies suggested that the polymerases with nascent 13-mers had properties of the arrested state (27, 28), which raised a possibility that the insensitivity of 13-mers to factor 2 action might be caused by the arrested state of the polymerase. To examine this possibility on more normal arrested complexes, an immobilized template was used to generate complexes arrested 112 base pairs downstream of the Act5C promoter (4, 8). These complexes are not able to elongate in the presence of 600 μM nucleotide triphosphates but are sensitive to DmS-II action (4). When these complexes were incubated with factor 2 in the presence of ATP, more than 60% of the transcripts were released (data not shown). The elongation complexes blocked at the same site were also observed to be sensitive to factor 2 action during elongation (Fig. 4B in Ref. 8). Thus, factor 2 can release transcripts associated with arrested elongation complexes. The insensitivity of 13-mer to factor 2 is likely caused by the physical state of DNA upstream of the elongation complex. Double-stranded DNA may be required for appropriate localization of the ATPase activity of factor 2 and may enable its coupling with transcript release. Since the elongation complexes formed on dC-tailed template were generated by pure RNA polymerase II, a particular localization of factor 2 in relationship to the elongation complex may be essential for a specific interaction between factor 2 and RNA polymerase.

How factor 2 activity is directed toward a subset of the total complexes is not understood. The transcript release activity of factor 2 per se does not require other protein cofactors. However, factor 2 may be modulated by other factors in the process of transcription elongation. An inhibitory activity of factor 2 was found to associate with low salt-washed early elongation complexes and protect a fraction of the complexes from being released by factor 2 (8). Furthermore, in reactions containing the factors required for the generation of DRB-sensitive long runoff transcripts, the appearance of DRB-sensitive runoff transcripts were suppressed by increasing the amount of purified factor 2 but not by a partially purified factor 2 fraction (6). This finding suggests that factor 2 has the ability to terminate the potentially productive elongation complexes that are going to generate DRB-sensitive runoff transcripts. The antitermination activity of factor 2 associated with the low salt-washed early elongation complexes or contained in the partially purified factor 2 fraction is likely required to restrict factor 2 from acting on the potentially productive elongation complexes. A preliminary result suggests that a 14-kDa protein that copurified with factor 2 through multiple steps, but was removed during chromatography on hydroxyapatite column, is required to support the production of DRB-sensitive runoff transcripts in the presence of purified factor 2 (2). Thus, during transcription elongation, the transcript release activity of factor 2 is likely to be regulated by other factors so that factor 2 preferentially acts on the elongation complexes that are not rescued by P-TEF.
to have an ATPase activity that can be activated by DNA. However, ERCC6 lacks the ability to terminate stalled RNA polymerase II (30). It was suggested that eucaryotes might utilize a different mechanism in transcription-coupled repair than *E. coli* in which the polymerase backs away from the damaged DNA due to the action of S-II (30, 31). It is not clear why such a difference should exist since similar transcript cleavage factors GreA and GreB exist in *E. coli* (32). In support of this idea, it has recently been shown that S-II is not required for transcription-coupled repair in yeast (33). We suggest the possibility that factor 2 may function as a TRCF, but further examination of the role of factor 2 in repair will be necessary to clarify its cellular function.

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