Communication

Purification of an RNA Polymerase II Transcript Release Factor from Drosophila*

(Received for publication, December 14, 1995, and in revised form, February 14, 1996)

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Factor 2 was previously identified in Drosophila Kc cell nuclear extract (KcN) as an activity suppressing the appearance of long transcripts (Price, D. H., Sluder, A. E., and Greenleaf, A. L. (1987) J. Biol. Chem. 262, 3244-3255). A 154-kDa protein with factor 2 activity was purified to apparent homogeneity from KcN. An immobilized template assay indicated that factor 2 caused the release of transcripts by RNA polymerase II in an ATP-dependent manner. Some early elongation complexes were resistant to factor 2 action but became sensitive after treatment with 1 M KCl. In the absence of factor 2, transcription complexes still exhibited a low degree of processivity suggesting that factor 2 was only partially responsible for abortive elongation.

The study of eucaryotic gene expression is aided by the application of procaryotic paradigms. A major feature of procaryotic transcriptional control is the default employment of negative elongation potential, which stops RNA polymerase before a gene is fully transcribed. Control of expression is achieved through the action of positive factors, which all RNA polymerase II molecules that initiate from a promoter are destined to produce only short transcripts due to the action of negative transcription elongation factors (N-TEF). Escape from this abortive elongation into productive elongation requires the action of positive transcription elongation factors (P-TEF). Fractionation studies have recently identified three components required to efficiently generate productive elongation complexes (15). One of these components, P-TEFb, was purified to apparent homogeneity and was shown to act after initiation (15). N-TEF was proposed to function early during transcription and result in pausing and premature termination (14). Here we report the purification of factor 2, a component of N-TEF, from Drosophila Kc cells.

EXPERIMENTAL PROCEDURES

Chromatography and Fractionation—General chromatography procedures were as described by Price et al. (16). All columns were run in HGKEDP (25 mM HEPES, pH 7.6, 15% glycerol, indicated molar concentration of KCl, 0.1 mM EDTA, 1 mM diithothreitol, and 0.1% of a saturated solution of phenylmethylsulfonyl fluoride in isopropyl alcohol) except ceramic hydroxyapatite column (Bio-Rad CHT10), for which potassium phosphate (pH 7.6) was used instead of KCl in HGKEDP (HGPEPD). Fractionation of KcN was carried out according to the step procedure described earlier (15).

Purification of Factor 2—Factor 2 was purified from Drosophila KcN using an in vitro transcription assay (see below). 124 ml of nuclear extract was loaded on a 500-ml P-11 column at 125 ml HKEPD. Proteins eluting between 0.1 and 0.3 M KCl were dialyzed against HGPEPD until the salt was 100 mM KCl and then loaded on a 250-ml DE52 column. Proteins bound to DE52 were eluted with a 100 to 600 mM KCl gradient. Factor 2 eluted from 180 to 220 mM KCl, and these fractions were pooled and directly loaded on a 10-ml ceramic hydroxyapatite column equilibrated with 50 mM HGPEPD. The material bound to the hydroxyapatite column was eluted with a 50-500 mM phosphate gradient. The fractions (220–350 mM phosphate) containing factor 2 were pooled and loaded on a 1-ml Mono Q column equilibrated at 160 mM KCl. The material bound to the Mono Q column was eluted with a 110–600 mM KCl gradient. Factor 2 eluted from 150 to 170 mM KCl and was pooled and loaded on a 1-ml Mono Q column equilibrated at 160 mM KCl. The material bound to Mono Q was eluted with a 160–700 mM KCl gradient, and factor 2 eluted from 320 to 340 mM KCl. A 500-μl sample from Mono Q fraction 36 (peak fraction of factor 2) was loaded on a 4.5-ml 20–35% glycerol gradient and centrifuged at 55,000 rpm (287,000 × gav) in a Beckman SW 55 Ti rotor at 1°C for 38 h.

In Vitro Transcription—A continuous labeling protocol (16) was used to assay the effect of factor 2 on transcription. Reactions contained the following: 20 mM HEPES, 5 mM MgCl2, 600 μM of each ATP, GTP, UTP, CTP, 30 μM CTP, 60 mM KCl, 3 μCi of [α-32P]CTP, 6 μg/ml DNA template, and partially purified factors that supported transcription initiation and elongation. The template was actin Ac5C (13), linearized with either Sall or Hpal giving a 1,000- or 520-nucleotide runoff, respectively. A mixture of partially purified factors included 0.2 μM of DNase inhibitor, 0.2 μM of RNA polymerase II, 0.2 μM of TFIIE (Drosophila factor 3), and 3.1 μl of concentrated P-11 (14) step. The reactions were started by adding a transcription mixture containing the buffer, MgCl2, NTPs, and template. 12.5-μl reactions were incubated at 25°C for 20 min. The reactions were stopped and the labeled transcripts were

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1 The abbreviations used are: N-TEF, negative transcription elongation factor(s); P-TEF, positive transcription elongation factor(s); TRCF, transcription/repair coupling factor; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

* This work was supported by National Institutes of Health Grant R01-GM35500. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
isolated and analyzed by denaturing gels (16).

Transcript Release Assay—The immobilized actin template was synthesized as described earlier (14). Transcription of the HpaII cut template generated a 780-nucleotide runoff. The template was first incubated with K,N in the presence of 20 mM HEPEs and 5 mM MgCl₂ for 10 min at 25°C to form preinitiation complexes. The preinitiation complexes were then pulse-labeled for 15 or 20 s to generate early elongation complexes. The pulse solution contained 5 μCi of [α-³²P]CTP and they were chased with HKE. The reaction mixture to 600 μM GTP, ATP, and UTP. The reaction was stopped by adding EDTA to a final concentration of 10 mM. The resulting early elongation complexes were concentrated magnetically and washed four times with HMK buffer, which contained 20 mM HEPEs, 5 mM MgCl₂, and either 60 mM, 250 mM, or 1 M KCl as described in the text. High salt washed complexes were washed another three times with low salt buffer. The washed early elongation complexes were resuspended in 60 mM HK buffer and aliquoted to individual reaction tubes (8 μl each). After addition of 4 μl of a mixture containing 60 mM HMK and the indicated concentration of ATP or factor 2, or both, the washed complexes were incubated at 25°C for 5 min. The reactions were stopped with 18 μl of HKE (20 mM HEPEs, 60 mM KCl, and 10 mM EDTA). The beads were concentrated, and the supernatant containing released transcripts was removed. Labeled transcripts in supernatant and bead fractions were isolated and analyzed by 18% denaturing gels (16). Labeled transcripts were quantitated using a Packard InstantImager.

To test the effect of factor 2 on early elongation complexes during transcription (see Fig. 4), a chase solution was added to allow further elongation in the presence or absence of factor 2. The chase solution brought the reaction to either 60 or 250 mM HMK and 600 μM GTP, ATP, CTP, and UTP. The reactions were stopped with HKE. The labeled transcripts in soluble and bead fractions were isolated and analyzed in 6% denaturing gels (16).

RESULTS

Elongation control involves the action of both positive and negative factors (13, 14). Recently we showed that partially purified Drosophila factor 2 was involved in this process (15). To further define the role of factor 2 we undertook its purification. We used a set of partially purified factors that reconcentrated accurately and washed four times with HMK buffer, which contained 20 mM HEPEs, 5 mM MgCl₂, and either 60 mM, 250 mM or 1 M KCl as described in the text. High salt washed complexes were washed another three times with low salt buffer. The washed early elongation complexes were resuspended in 60 mM HK buffer and aliquoted to individual reaction tubes (8 μl each). After addition of 4 μl of a mixture containing 60 mM HK buffer and the indicated concentration of ATP or factor 2, or both, the washed complexes were incubated at 25°C for 5 min. The reactions were stopped with 18 μl of HKE (20 mM HEPEs, 60 mM KCl, and 10 mM EDTA). The beads were concentrated, and the supernatant containing released transcripts was removed. Labeled transcripts in supernatant and bead fractions were isolated and analyzed by 18% denaturing gels (16).

Using this system factor 2 suppressed the appearance of long transcripts (16).

Partially Purified Factor 2 Contains a Transcript Release Activity—We examined the properties of factor 2 at an intermediate stage in its purification. A third column fraction (Mono Q, see “Experimental Procedures”) was titrated into recombinant reactions lacking P-TEFb. As the amount of factor 2 increased the appearance of long transcripts was suppressed (Fig. 1A). To exclude the possibility of ribonuclease contamination, a transcript reaction was carried out without factor 2 and then stopped with α-amanitin. The reaction was then incubated with or without partially purified factor 2 for another 10 min. No change was observed in the pattern of transcripts indicating that the crude factor 2 fraction was not contaminated with ribonuclease (Fig. 1A, last two lanes).

Since factor 2 suppressed the generation of long transcripts, increased pausing or termination was a possible explanation. An immobilized DNA template was used to differentiate paused from terminated transcripts. Early elongation complexes were isolated and then incubated with partially purified factor 2 in the presence or absence of ATP. Released RNAs were separated from the template-associated transcripts by magnetic concentration. Factor 2 or ATP by itself was not sufficient to release the transcripts associated with early elongation complexes (Fig. 1B). However, in the presence of ATP, partially purified factor 2 caused the release of transcripts associated with the immobilized complexes (Fig. 1B).

Factor 2 is a 154-kDa Monomer—To further characterize the factor we used the continuous labeling transcription assay and the transcript release assay to purify factor 2. After multiple steps (see “Experimental Procedures”), factor 2 was purified to near homogeneity. Analysis of the fifth and sixth purification steps (see “Experimental Procedures”), factor 2 was purified to near homogeneity. Analysis of the fifth and sixth purification steps, Mono Q chromatography and glycerol gradient sedimentation, showed that a 154-kDa protein correlated with factor 2 activity in both assays (Fig. 2). Comparison of the sedimentation of factor 2 to known proteins suggested that factor 2 was a monomer (data not shown).

Factor 2 Associates with Elongation Complexes—Early elongation complexes washed with either 60 mM or 1 M HMK were tested for their dependence on ATP or factor 2 to release nascent transcripts (Fig. 3A). The low salt washed complexes released 40% of the associated transcripts when only ATP was added, and further supplementation of factor 2 had little effect (Fig. 3A). This transcript release activity could be due to associated factor 2 or another ATP-dependent transcript release factor. Complexes washed with 1 M HMK were not able to release nascent transcripts when only ATP was added. Therefore, the ATP-dependent transcript release activity associated with low salt washed complexes was removed by the high salt wash. However, when factor 2 was supplemented in the presence of ATP, 70% of the RNAs associated with low salt washed complexes were released (Fig. 3A). It is possible that the high salt wash removed protein(s) that inhibit transcript release. A comparison of the pattern of transcripts released indicated that there was a slight preference for retention of longer transcripts (over 19 nucleotides) by low salt washed complexes while transcripts of all sizes were equally released from high salt washed complexes. This difference may be due to heterogeneity of the low salt washed complexes. If there was an inhibitor of transcript release, it might preferentially associate with complexes containing longer transcripts.

Factor 2 promoted transcript release from high salt washed complexes, but it was not clear if it associated with these complexes. To address this question, high salt washed early elongation complexes were generated and incubated with factor 2 without ATP. After washing again with low salt, these complexes were able to release 60% of the associated transcripts when only ATP was added. This indicated that factor 2
stably associated with the elongation complexes under low salt conditions. Supplementation with additional factor 2 caused a slight increase in the percentage of transcripts released (Fig. 3B). This additional release could have been due to a subsaturating level of factor 2 being used in the first incubation. After washing again with 1 M KCl, most of the transcript release activity was removed, indicating that the association of factor 2 with elongation complexes was not stable to high salt (Fig. 3B). Supplementation of factor 2 restored the transcript release activity. These association studies indicated that the properties of factor 2 were similar to those of the transcript release activity found in the initial low salt washed complexes (Fig. 3A). The simplest explanation is that factor 2 is responsible for all transcript release we observed. Once antibodies to factor 2 are available, it will be possible to determine if factor 2 is present in the initial elongation complexes.

Factor 2 Promotes the Release of RNAs from Transcription Complexes during Elongation—All of the transcript release experiments shown so far utilized elongation complexes stalled by depleting the NTPs. The continuous labeling experiments (Fig. 2, B and E) indicated that factor 2 functioned during elongation. To determine if factor 2 could cause the release of transcripts during elongation, we examined the effect of factor 2 on isolated complexes supplemented with NTPs. As was found with stalled complexes, 40% of the associated transcripts were released during elongation by low salt washed complexes (Fig. 4A). Similarly, more than 50% of the transcripts were resistant to release during elongation, even when supplemented with factor 2. These results suggested that the hypothesized inhibitor of transcript release did not dissociate during elongation. Even though the resistant complexes remained competent to elongate in the presence of high salt, elongation under low salt was limited. This suggested that some component of N-TEF was present (Fig. 4A).

As expected, high salt washed early elongation complexes did not release transcripts during subsequent elongation in 60 mM KCl. Surprisingly, these complexes still encountered blocks to elongation (Fig. 4B). These blocks were substantially relieved by chasing with 250 mM KCl. Evidently, some component of N-TEF seen in low salt washed resistant complexes was retained after the high salt wash. This component of N-TEF was apparently suppressed but not removed by treatment with high salt. When the high salt washed complexes were supplemented with increasing amounts of factor 2, there was a decrease in the length of the transcripts synthesized and an increase in the amount of released transcripts (Fig. 4B). As was seen with the stalled complexes, almost all of the complexes became substrates for factor 2 after high salt treatment.

DISCUSSION

We purified Drosophila factor 2 and determined that it caused the release of the RNA component of RNA polymerase II elongation complexes in an ATP-dependent manner. We refer to factor 2 as a transcript release factor rather than a termination factor only because we have not determined if the polymerase is also released from the template. Since it is not likely that the polymerase continues to synthesize RNA after the transcript is released, it is probable that factor 2 acts as a true termination factor.

Factor 2 is involved in abortive elongation but is not completely responsible for the process. Abortive elongation is characterized by the rapid generation of short transcripts due to pausing of the polymerase followed by termination of some of the transcripts (13, 14). Although factor 2 plays a role in transcript release, two of our results indicate that other factors also contribute to abortive elongation. First, the low salt washed complexes that were resistant to factor 2 were unable to synthesize long transcripts at 60 mM KCl even though the transcripts remained in elongation complexes (Fig. 4A). Second, complexes washed by high salt, though lacking factor 2, still synthesized shorter transcripts on average at 60 mM KCl compared with 250 mM KCl (Fig. 4B). It appeared that some of the abortive properties of the elongation complexes could be suppressed but not removed by high salt. Our earlier results (13, 14) and those presented here suggest that preinitiation complexes confer a negative elongation potential on the early elon-
showntostimulateproductiveelongationbyP-TEFb(15).Pre-
typeofelongationcomplex. Partially purified factor 2 was
specific factor restricting the activity of factor 2 to a certain
proteinthatblockstheassociationoffactor2orcouldbea
ctionoffactor2inelongationcontrol.

involved will be necessary to completely understand the func-
tion of the inhibitor and identification of other factors that may be
removed by the low salt wash. Elucidation of the properties of
factor 2 or the inhibitor, and these factors would have been
continuouslabelingassaymighthaveinfluencedtheactivityof
washing. Factors, including known elongation factors, in the
plexeshavebeenresistantto transcriptrelease. This was demon-
strated by the inability of added factor 2 to cause additional
plexescrasheduringelongation. The lack of function of factor 2
on isolated early elongation complexes (Fig. 3A) seemed to con-
dict the clear effect of added factor 2 in the continuous labeling
transcription experiments using crude fractions (Fig. 2, B and E).
This apparent discrepancy arose because the two assay
systems were quite different. In the transcript release assay
The inhibitor of factor 2 function could be a nonspecific
protein that blocks the association of factor 2 or could be a
specific factor restricting the activity of factor 2 to a certain
type of elongation complex. Partially purified factor 2 was
shown to stimulatethe appearanceof P-TEFb (15). Prelim-
inary results with pure factor 2 suggest the stimulatory
effect was probably due to contaminating factors in the fraction
(data not shown). In reactions containing the factors required
for the generation of DRB-sensitive long runoff transcripts, the
crude factor 2 fraction used did not inhibit the appearance of
long transcripts (15), suggesting that factor 2 selectively acted
on a subset of elongation complexes. One intriguing possibility
is that the inhibitor protects a subset of elongation complexes
from factor 2 action and that these complexes are then acted
upon by P-TEF to allow the transition into productive elonga-
tion. Alternatively, it is possible that only the complexes that
factor 2 can act on are potentially productive. If this is the case,
P-TEF would have to act before factor 2 caused transcript
release. These opposing models could be tested by determining
if P-TEF can act on the factor 2-resistant complexes.

The mechanism of factor 2 action may share some similarities
with other termination factors. Factor 2 is like the E. coli
rho factor and vaccinia capping enzyme in that it requires ATP
for function (7, 17). Unlike rho, however, factor 2 can associate
with early elongation complexes containing RNA less than 10
nucleotides in length and cause transcript release. Such short
transcripts are probably still sequestered within RNA polymer-
ase II (18, 19) and are not accessible to RNA binding proteins.
The tight interaction of factor 2 with early elongation com-
plexes is more likely through DNA, RNA polymerase II itself,
or other associated factors. A transcription/repair coupling fac-
tor (TRCF) has been identified in E. coli, which increases the
rate of repair of transcribed regions (20, 21). TRCF recognizes
stalledelongationcomplexescausedbyDNAlesions,nucleo-
tide starvation, or protein roadblocks and dissociates the ter-
nary complexes upon ATP hydrolysis. TRCF contains an RNA
polymerase binding motif and binds double strand DNA but
has little affinity for RNA. There is a possibility that factor 2
may be the eucaryotic homologue of E. coli TRCF, but protein
sequence and detailed functional studies are needed to justify
this hypothesis.

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