PIOTR DULLIN, MARJOLA GALBAS, GRAŻYNA CHODYNICKA-KORDUS, ANNA FABISZ-KIJOWSKA and WITOLD WALERYCH

TRANSCRIPTIONAL ACTIVITY OF A COMPLEX OF WHEAT RNA POLYMERASE II AND NONHISTONE CHROMOSOMAL PROTEIN (NHCP) *

Institute of Biochemistry, Agriculture University, Wołyńska 35; 60-637 Poznań, Poland

Received 24 May, 1989

A protein stimulating RNA polymerase activity that has been isolated and partially characterized previously (Wastowicz, W., Fabisz-Kijowska, A. et al., 1982, Phytochemistry, 21, 1495-1507) was subjected to investigations on its possible involvement in transcription process.

Affinity chromatography revealed that this protein strongly binds to RNA polymerase-Sepharose column. The NHCP protects the enzyme against thermal inactivation, while the polymerase stabilizes the NHCP and maintains its ability to stimulate DNA transcription. The NHCP increases the affinity of RNA polymerase to DNA. Upon its addition, longer transcripts are obtained when a zein gene is used as a template for in vitro transcription.

The RNA polymerase-NHCP complex formed has greater affinity to DNA than free enzyme. This seems to be due to enhanced sensitivity of bound enzyme to signals encoded in DNA sequences.

RNA polymerase is an enzyme that mediates the transcription process. Purified RNA polymerase is incapable of initiating specific transcription and requires supplementation with other cellular substances. Several protein factors involved in accurate transcription initiation have been discovered [1 - 5] and it has been suggested that formation of the initiation complex is a multistep process [2].

---

1 NHCP, nonhistone chromosomal protein; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; NTP, nucleoside triphosphate.

* This work was supported by the Polish Academy of Sciences, grant CPBR 3.13.1.2.12 and CPBP 04.12.1.6.
Much research has been carried out on the initiation of transcription in comparison with studies on the elongation of the RNA chain. Several studies on the elongation process pointed to the existence of a protein able to stimulate RNA polymerase random transcription [3, 6-10].

Among the nonhistone proteins loosely bound to wheat germ chromatin we found some proteins which stimulated transcription [10]. We also assume that RNA polymerase in the presence of this protein factor produces longer RNA chains when compared with the transcription in its absence [11].

Recently our efforts have been concentrated on determining mechanisms which are responsible for the synthesis of longer RNA chains in the presence of nonhistone transcription stimulatory protein.

MATERIALS AND METHODS

Chemicals and radiochemicals. Polymin P was purchased from BASF. Ammonium sulphate (ultrapure), PMSF and DTT were purchased from Merck. All unlabelled nucleoside triphosphates were obtained from Sigma. Sephadex (G-100, DEAE-A 25, G-200), CNBr-Sepharose were purchased from Pharmacia. [3H]CTP was from Amersham.

Preparation of RNA polymerase. Wheat germs (Triticum aestivum) were obtained from the local mill.

The initial steps of preparation of the RNA polymerase II (isolation, extraction, precipitation with Polymin P, ammonium sulphate precipitation and DEAE-Sephadex chromatography) were carried out as reported by Fabisz-Kijowska et al. [12] essentially according to [13]. The ammonium sulphate precipitate was dissolved in 0.05 M Tris/HCl buffer, pH 8.0, containing 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF and 25% glycerol (buffer A), dialysed and applied to a DEAE-Sephadex column. The proteins were recovered from the column by ammonium sulphate stepwise elution (0.1, 0.15, 0.23, 0.25, 0.35, 0.5 M). The fractions containing RNA polymerase activity were collected, dialysed against buffer A, and concentrated by dialysis against buffer A containing 50% glycerol. The active fractions were chromatographed on a Sephadex G-100 column equilibrated with buffer A containing 25% glycerol. Fractions showing RNA polymerase activity were combined, dialysed against buffer A and concentrated by dialysis against buffer A containing 50% glycerol.

RNA polymerase IIA was separated from RNA polymerase IIB by chromatopography on DEAE-Sephadex (form IIA was eluted at 0.23 M ammonium sulphate concentration while form IIB was eluted at 0.25 M ammonium sulphate).

Determination of DNA dependent — RNA polymerase II activity. The activity of RNA polymerase was estimated according to [12] in the standard
incubation mixture (final volume of 20 µl) containing: 50 mM Tris/HCl buffer, pH 8.0, 10 mM MgCl\textsubscript{2}, 1 mM MnCl\textsubscript{2}, 1 mM DTT, ATP, GTP, UTP each at 0.25 mM concentration, 1 µCi [\textsuperscript{3H}]CTP, 1 µg double-stranded wheat germ DNA and purified RNA polymerase II (0.05 - 0.2 µg). The mixture was incubated for 10 min at 30°C. The reaction was terminated by addition of equal volume of 10% trichloroacetic acid.

The radioactivity of the precipitate insoluble in 5% trichloroacetic acid was measured in Beckman LS 100 liquid scintillation counter.

Isolation of transcription stimulatory protein. The transcription stimulatory protein was isolated from wheat germ chromatin [10] and chromatographed on a DEAE-Sephadex A-25 column [11].

Determination of RNA synthesis initiation. This was determined in the same way as estimation of RNA polymerase activity with the exception that [\textsuperscript{γ-32P}]ATP was used as a labelled substrate.

Thermal inactivation analysis. Thermal inactivation of the RNA polymerase or NHCP was carried out in the standard incubation mixture without nucleoside triphosphates, DNA and NHCP or RNA polymerase, respectively. Inactivation of the RNA polymerase and NHCP was assayed at 52 and 45°C, respectively. After the incubation period, the omitted compounds were added and residual activity of RNA polymerase, or the transcription stimulatory activity were determined.

Preparation of affinity media. RNA polymerase-Sepharose was prepared by the method of Sopta et al. [9]. For blocking the active group on CNBr-Sepharose, aminoacetic acid replaced etanolamine used in the original protocol.

DNA-celullose was prepared by method described in [14, 15] using wheat germ DNA.

Affinity chromatography of NHCP. The same NHCP fraction, 5 mg, in 10 ml of buffer A containing 10 mM MgCl\textsubscript{2}, 1 mM MnCl\textsubscript{2} and 25% glycerol was slowly applied (0.1 ml/min) on RNA polymerase-Sepharose or DNA-celullose column. Both columns were equilibrated with the above buffer. The proteins were recovered from the column by NaCl stepwise elution (0.0, 0.2, 2 M); 1.5 ml fractions were collected and stimulation of transcription activity was estimated.

Protein concentration. This was determined by the method of Bradford [16] using bovine serum albumin as a standard.

Transcription and gel electrophoresis of transcript. The transcription was carried out for 30 min in incubation mixture containing in 50 µl: 1 µg of the PML\textsubscript{1} plasmid with zein gene (a kind gift from prof. G. Feix, Frieburg) and [\textsuperscript{α-32P}]UTP. The reaction was stopped by the addition of sodium acetate and SDS to the final concentration of 0.2 M and 0.5% respectively, Nucleic acids were precipitated by ethanol.

The transcription product was analyzed in 5% polyacrylamide gel with
8 M urea in 50 mM Tr's/borate buffer, pH 8.3, with 1 mM EDTA [17]. Autoradiography of the gel was made with Fotopan X-ray film.

RESULTS

The transcription stimulating effect of nonhistone protein on the kinetics of incorporation of nucleoside monophosphates into RNA was studied.

Nucleoside triphosphates are utilized as a substrate by RNA polymerase II in two types of reaction: initiation and elongation of the RNA chain. ATP and GTP are required for the initiation step, but all four nucleoside triphosphates are used in the elongation step. We studied the effect of NHCP on RNA elongation kinetics using CTP as a labelled substrate. When the effect of the stimulatory protein was evaluated at varying CTP concentrations double reciprocal plots showed that with the addition of NHCP both $K_m$ and $V_{max}$ were changed (Fig. 1).

![Fig. 1. Kinetic analysis of the effect of NHCP on transcriptional activity of RNA polymerase at variable CTP concentration. The activity of RNA polymerase was determined as described in Materials and Methods. The samples were incubated for 10 min without NHCP (control, ●) or with the addition of 0.1 μg of NHCP (○). The results are mean values of triplicate assays from two separate experiments.](image)

Analysis of the effect of NHCP protein on the kinetics of $[^3H]\text{CMP}$ incorporation at variable DNA concentrations showed no change in $V_{max}$ value, whereas affinity of RNA polymerase to the template markedly increased (Fig. 2). This suggests that the transcription stimulatory protein affects the binding of RNA polymerase to DNA.

In Table 1 are presented the results of preincubation of RNA polymerase with NHCP and DNA. NTP were added at the beginning of incubation. This means that preincubations were conducted without NTP. Experiment No. 1 is a standard incubation mixture. Experiments No. 3, 7 and 9 were
Fig. 2. Kinetic analysis of the effect of NHCP on transcriptional activity of RNA polymerase at variable DNA concentration. The activity of RNA polymerase was determined as described in Materials and Methods. The samples were incubated for 10 min without NHCP (control, ●) or with the addition of 0.1 μg of NHCP (○). The results are mean values of triplicate assays from two separate experiments.

Table 1

Effect of preincubation of RNA polymerase II, DNA and NHCP on enzyme activity

Preincubation was carried out in the mixture containing all compounds of the standard incubation mixture with exception of NTP's (as described in Materials and Methods); +, added compound; −, omitted compound

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Preincubation 5 min, 30°C</th>
<th>Incubation 10 min, 30°C</th>
<th>RNA polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>enzyme</td>
<td>DNA</td>
<td>NHCP</td>
</tr>
<tr>
<td>1.</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2.</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3.</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4.</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7.</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>8.</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>9.</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10.</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
carried out without NHCP. Estimation of the effect of preincubation on the RNA polymerase II activity shows that this enzyme is stimulated by NHCP in all combinations tested (Expts. 2, 5, 6 and 8). No polymerase stimulation was observed when NHCP was preincubated alone or with DNA. This suggests that NHCP forms a complex with RNA polymerase. When NHCP was added after preincubation of the enzyme with the template, its stimulatory effect was lower than when it was added to the preincubation mixture (Expt. 8). This suggests that the stimulatory protein is unable to bind to the polymerase-DNA complex. This implies that RNA polymerase interacts with this protein first and that the ternary complex is formed later.

Estimations of the effect of NHCP on kinetics of RNA synthesis initiation, carried out with different concentrations of DNA (Fig. 3), and $[^{32}P]ATP$ (Fig. 4) showed that NHCP did not affect the initiation of RNA synthesis.

![Graphs](image)

**Fig. 3.** Kinetic analysis of the effect of NHCP on the initiation of RNA synthesis at variable DNA concentrations. The initiation of RNA synthesis was determined as described in Materials and Methods. The samples were incubated for 10 min without NHCP (control, ◦) or with the addition of 0.1 μg of NHCP (○). The results are mean values of triplicate assays.

**Fig. 4.** Kinetic analysis of the effect of NHCP on the initiation of RNA synthesis at variable $[^{32}P]ATP$ concentrations. The initiation of RNA synthesis was determined as described in Materials and Methods. The samples were incubated for 10 min without NHCP (control, ◦) or with the addition of 0.1 μg of NHCP (○). The results are mean values of triplicate assays from two separate experiments.
For binding of stimulatory proteins, an RNA polymerase 0.2 M NaCl stimulatory fraction — Sepharose column was used. The observed binding stimulatory fraction to this column (Fig. 5) points to its affinity to RNA polymerase. On the other hand, in an analogous experiment with the use of DNA-cellulose NHCP showed no affinity to this ligand (Fig. 6). Thus, results of affinity chromatography demonstrated that the transcription stimulatory protein shows affinity to RNA polymerase but not to the template.

![Fig. 5. Affinity chromatography on RNA polymerase-Sepharose of NHCPs from wheat germ. NHCPs were eluted from the column by stepwise gradient of NaCl as described in Materials and Methods. ○, activity of RNA polymerase with 5 μl of fraction; ———, A₂₈₀nm.](image)

![Fig. 6. Affinity chromatography on DNA-cellulose of NHCP from wheat germ. NHCPs were eluted from the column by stepwise gradient of NaCl as described in Materials and Methods. ○, activity of RNA polymerase with 5 μl of fraction; ———, A₂₈₀nm.](image)

Thermal inactivation experiments were conducted at 45°C for NHCP and 52°C for RNA polymerase. Optimal conditions for these assays were determined experimentally. RNA polymerase was protected by both DNA and the stimulatory protein (Fig. 7), whereas the ability of NHCP to stimulate transcription was supported by RNA polymerase but not by DNA (Fig. 8).

The effect of NHCP on the elongation step of transcription was studied with PML₁ plasmid, bearing zein gene, as a template in the presence of ApG (Fig. 9). In the absence of transcription stimulatory protein and ApG a few RNA bands were formed. In the presence of NHCP
Fig. 7. Thermal inactivation of RNA polymerase. The thermal inactivation of RNA polymerase was carried out as described in Materials and Methods. The samples of RNA polymerase were incubated without DNA, NHCP and NTP (control, ●) or with NHCP (△) or DNA (○). The conditions of the inactivation were the same except for variable time of incubation.

Fig. 8. Thermal inactivation of NHCP. The thermal inactivation of NHCP was carried out as described in Materials and Methods. The samples of NHCP were incubated without DNA, RNA polymerase and NTP (control, ●) or with RNA polymerase (○) or DNA (△). The conditions of the inactivation were the same except for variable time of incubation.
the number and intensity of bands markedly decreased. The longest RNA chains were about 1300 and 800 bases long.

DISCUSSION

In our earlier publications we presented isolation, purification and some properties of the nonhistone factor which stimulates transcription [10, 11]. The highest stimulation of the nonspecific transcription was obtained when we used polymerase IIB as an enzyme and wheat double-stranded DNA as a template. This nonhistone factor led to the synthesis of longer RNA chain [11] and its molecular weight and isoelectric point were similar to those of the SII factor isolated from Erhlich ascites cells [6].

![Fig. 9. Gel electrophoresis of transcript. The transcription was carried out as described in Materials and Methods. The reaction was stopped after 30 min of incubation. Electrophoresis of the transcript was carried out in 5% polyacrylamide gel. Lanes 1 - 5 contain, in incubation mixture, Polymerase IIA; lanes 6 - 10, polymerase IIB; lanes 1, 6, polymerase alone; lane 2, 7, polymerase +0.2 μg of NHCP; lanes 3, 8, polymerase +0.4 μg of NHCP; lanes 4, 9, polymerase +0.2 NHCP and ApG; lanes 5, 10, polymerase with 0.4 μg NHCP and ApG. Arrows indicate RNA standards and main RNA band (800 and 1300 bases).](image)
At present we are focusing our attention on determining the mode of action of this protein. The results obtained so far suggest that this factor forms a complex with RNA polymerase II because: (1) during affinity chromatography it remains bound to RNA polymerase-Sepharose on the column, (2) it is protected by this enzyme during thermal inactivation, (3) it protects the polymerase during thermal inactivation, (4) it changes the affinity of the enzyme to the template and substrates.

Affinity chromatography on RNA polymerase-Sepharose revealed certain similarities of this factor to proteins isolated by Supta et al. [9]. The change of affinity of the enzyme to the template in the presence of this protein could also suggest formation of a complex between the stimulatory protein and DNA, and that such complex could enhance the affinity of RNA polymerase to the template "modified" in this way. This possibility however was excluded by the results of affinity chromatography on cellulose with DNA as a ligand. In the experimental conditions applied, NHCP was not retained on the column. Roeder et al. [1 - 3] in a series of experiments used ss DNA-agarose for purification of the transcription factors (TF IIB, TF IIID, TF IIS). Initiating factors (IF IIB and D) bound to ss DNA-agarose [1, 2]. On the other hand, TF IIS, which stimulates elongation, did not bind to such ligands. This indicates that there is a similarity between TF IIS and the transcription stimulatory protein isolated from wheat germ. That the stimulatory protein-DNA complex was not formed was further confirmed by the fact that this protein was not protected by DNA during thermal inactivation.

During chromatography on the RNA polymerase-Sepharose column as well as during thermal inactivation of RNA polymerase and NHCP the complex of NHCP-RNA polymerase was formed without NTP, which indicates that this reaction does not require energy.

It seems that the transcription stimulatory factor isolated from plant tissue is very similar to the elongation stimulatory proteins which were isolated from tumour cells by Natori [6] and Roeder groups [2].

While analyzing the effect of these proteins on the kinetics of transcription initiation, we did not observe any changes which would suggest their involvement during this stage of transcription. Reinberg & Roeder [2] came to the same conclusion when analyzing the influence of TF IIS protein in the presence of heparin initiation inhibitor.

Analysis of the effect of the plant transcription stimulatory factor showed that both in the case of nondefined [11] and defined template (Fig. 9) the presence of this factor in the incubation mixture leads to the synthesis of longer RNA chains. During the transcription of defined templates, shorter DNA chains disappear. It is possible that this factor acts as an antiterminator described by Maderious & Chen-Kiang [18]. The mode of action of all proteins which stimulate elongation of the polycytoplasmic
acid chain is hitherto unknown. There are suggestions that phosphorylation of this factor constitutes a significant element of their action [8, 19]. Kinetic data indicate increased affinity to DNA of the polymerase in a complex with NHCP. It is known that the information encoded on the template concerns not only the sequence of bases in RNA but also contains signals concerning transcription initiation and termination [20]. It is conceivable that shorter DNA chains are formed as the result of termination in places in which the sequence is similar to that of normal terminating places. The reduced frequency of termination in those places may, in turn, be the outcome of the increased affinity of the enzyme in the complex with NHCP to the template and, consequently, higher precision of decoding of signals which were encoded on this template.

The transcription stimulatory protein factor isolated from the chromatin of wheat germs forms a complex with RNA polymerase irrespective of the presence or absence of DNA. However, this factor is not active at the initiation step of transcription but at the later stages.

We are extremely grateful to Prof. G. Feix for providing us with PML₁ plasmid.

REFERENCES


