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PREFERENTIAL STIMULATION OF RNA POLYMERASE II_B BY A CHROMOSOMAL PROTEIN FROM WHEAT GERM

ANNA FABISZ-KIJOWSKA, PIOTR DULLIN, RYSZARD CZAPARA, WITOLD WALERYCH, BLANKA SZURMAK* and JERZY BUCHOWICZ*

Institute of Biochemistry, Agriculture University, 60–637 Poznań, Poland; *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02–532 Warsaw, Poland

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Key Word Index—*Triticum aestivum*; Gramineae; wheat embryo; transcription regulation; non-histone chromosomal proteins; RNA polymerases; RNA polymerase II stimulatory protein; RNA polymerase II_B.

Abstract—An RNA polymerase II stimulatory protein was purified from wheat germ chromatin and tested for the ability to stimulate wheat germ RNA polymerases I, II_A, II_B and III. The form II_B of the class II enzyme was stimulated most effectively. The stimulation of [^{14}C]GTP incorporation was accompanied by an increase in the RNA product size, especially if native wheat germ DNA was used as template. The stimulatory protein was also able to reverse inhibition of transcription caused by other chromosomal proteins, and was a particularly preferred substrate for cyclic AMP-independent wheat germ protein kinase. The kinase preparation, however, lost its stimulatory activity. It is concluded that a form II_B-specific RNA polymerase II stimulatory protein occurs and may be involved in the regulation of transcription in wheat embryo cell nuclei.

INTRODUCTION

RNA polymerase II may be obtained in two chomatographically distinct forms, II_A and II_B, that differ from each other in the size of their largest subunits (MWs *ca* 220 000 and 180 000, respectively; for review and nomenclature, see ref. [1]). It remains to be elucidated, however, whether this structural difference has any physiological significance. Indirect evidence seems to indicate that these two enzyme forms do have distinct functions in cell nuclei. In higher plants, for example, form II_A is known to predominate quantitatively in resting tissues [2, 3]. In rapidly growing tissues, however, and in response to growth stimulating factors in particular [3], the ratio changes in favour of RNA polymerase II_B activity implying that this enzyme form, rather than RNA polymerase II_A, may be directly involved in the nuclear genome transcription *in vivo*. If so, differences in response of the enzyme forms II_A and II_B to RNA polymerase II regulatory proteins would also be expected.

To observe such differences, we have chosen an NHCP fraction of wheat germ, that has previously been shown to contain an RNA polymerase II stimulatory factor [4], as a source of the regulatory protein. As described below, wheat germ RNA polymerase II_B differs from the enzyme form II_A in its response to the stimulatory protein.

Abbreviations: NHCP, non-histone chromosomal protein(s); NHCP-1b, -1e and -1eII, NHCP subfractions obtained as described in text; TEDGP, 50 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol, 25% (v/v) glycerol and 0.1 mM phenylmethylsulphonyl fluoride; TMg, 20 mM Tris–HCl (pH 7.5) and 10 mM $MgCl_2$.

RESULTS

Separation of the wheat germ RNA polymerase activities

A simple purification procedure (see Experimental) was used to isolate total RNA polymerase activity from wheat germ. The total RNA polymerase preparation was then fractionated by chromatography on a DEAE-Sephadex column. Four discrete peaks (I, II, II′ and III) of RNA polymerase activity were observed (Fig. 1). The order of their elution and sensitivity to α-amanitin (Fig. 2) indicate that peak I, II, II′and III enzymes correspond most probably to RNA polymerases I, II_A, II_B and III, respectively.

A summary of the purification of wheat germ RNA polymerases is given in Table 1. The form II_A of the class II enzyme predominated, apparently, among the wheat germ RNA polymerase species and was isolated as a preparation of the highest total activity. Its specific activity is nearly 10-fold higher than that reported by Jendrisak and Burgess [5] for a highly purified RNA polymerase II (form II_A mainly, if not exclusively) preparation of the same source. The enzyme form II_B has not been purified previously from dry embryos.

The enzyme forms II_A and II_B showed essentially the same requirements, typical for the class II enzyme, for their catalytic activity. Both forms required the presence of Mg^{2+} and Mn^{2+} ions (at concentrations used to prepare standard RNA polymerase assay, see Experimental) for maximal activity and were inhibited by α-amanitin to a similar extent (see Fig. 2). The only difference was in their abilities to transcribe various DNA templates. As summarized in Table 2, form II_B showed a strong preference for homologous DNA and was able to

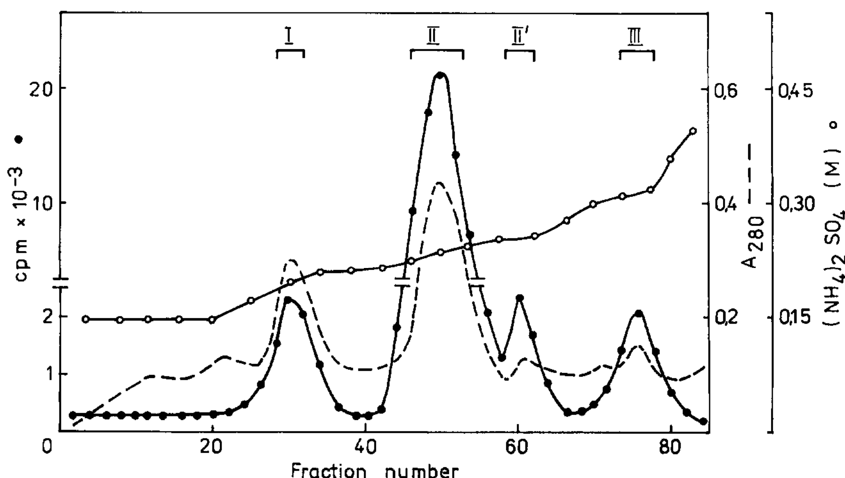

Fig. 1. Separation of wheat germ RNA polymerase activities by chromatography on a DEAE-Sephadex A-25 column. Purification step 4 protein (see Table 1) was applied to the column (0.9 × 30 cm) in TEDGP + 0.15 M ammonium sulphate and eluted with a salt gradient. Fractions of 3 ml were collected and, in 20-μl portions, assayed for RNA polymerase activity. The concentration of ammonium sulphate was measured conductimetrically. Fractions within each of the enzyme activity peaks (I, II, II' and III) were pooled (as the brackets show) to give the corresponding RNA polymerases (I, II_A, II_B and III, respectively).

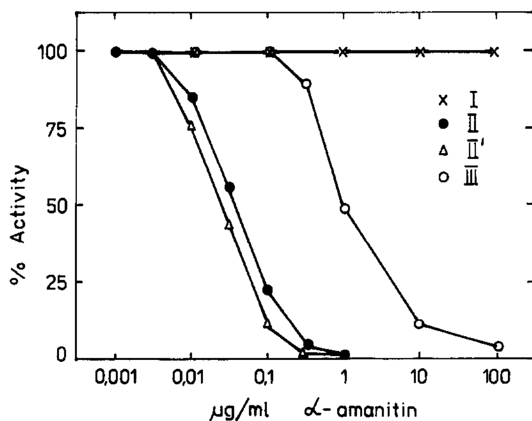

Fig. 2. Effect of α-amanitin on the activity of the isolated RNA polymerase species. Enzymes separated by DEAE-Sephadex CC (see Fig. 1) were assayed at the given concentrations of α-amanitin. Activities of 100% (in the absence of the toxin) were 16.7, 51.2, 31.6 and 9.2 pmol of GMP incorporated/assay for peak I, II, II' and III enzymes, respectively.

transcribe native wheat germ DNA more efficiently than did form II_A.

SDS-polyacrylamide gel electrophoresis showed RNA polymerase II_A to be composed of 11 subunits of various MW, including a component having MW of 220 000 as the largest polypeptide. A similar electrophoretic pattern was found also for the enzyme form II_B subunits, except for the absence of the band corresponding to a MW of 220 000 and the presence of one corresponding to a MW of 180 000.

Isolation of a form II_B-specific RNA polymerase II stimulatory protein

The previously described RNA polymerase II stimulatory protein NHCP-1e of wheat germ chromatin [4] was subjected to further purification by DEAE-Sephadex CC. The stimulatory activity eluted as two peaks of similar height (Fig. 3). The activity of the peak II was, however, associated with a much smaller amount of protein indicating better purification. Fractions corresponding to the peak II were, therefore, pooled and, after dialysis, lyophilized. The product obtained, referred to as NHCP-1eII, was found to stimulate RNA polymerase II_B more efficiently than any other RNA polymerases tested, including form II_A of the class II enzyme (Fig. 4). As little as 0.3 μg of the stimulatory protein (per 0.2-ml assay) was sufficient to achieve a 2.5-fold stimulation of RNA polymerase II_B activity and the stimulation increased over this value at higher NHCP-1eII concentrations. As found by SDS-polyacrylamide gel electrophoresis, the NHCP-1eII preparation contained two polypeptide components of MWs 55 000 and 37 000, respectively.

The overall purification procedure is summarized in Table 3. An increase in the total activity of the isolated product indicates that its purification is accompanied by elimination of proteins which, apparently, inhibit RNA polymerase activity (cf. Table 4). It was probably the presence of such proteins that made it difficult to detect the stimulatory activity in unfractionated chromatin and in the crude salt extract.

Mechanism of enhanced RNA synthesis

In attempts to elucidate whether the transcription stimulation observed was at the initiation or elongation step, the effect of NHCP-1eII on transcript length was investigated. A series of preliminary experiments indicated that, independently of the source (calf thymus, wheat and rye germs) and form (native and denatured) of DNA used as template, most of the transcripts formed by RNA polymerase II_B in the absence of NHCP-1eII corresponded in size to products slightly larger than tRNA. In the presence of NHCP-1eII, however, the

Table 1. Summary of purification of wheat germ RNA polymerases

Purification step	Volume (ml)	Protein (mg)	Total activity (units*)	Specific activity (units/mg)	RNA polymerase identified
1. Crude extract	450	11 070	132.8	0.01	—
2. Polymin P	200	960	74.9	0.08	—
3. $(NH_4)_2SO_4$	15	120	28.8	0.24	—
4. DEAE-Sephadex batch	100	20	66.6	3.33	—
5. DEAE-Sephadex CC,					
peak I	18	1.1	9.9	9.00	I
peak II	24	2.0	51.0	25.50	II_A
peak II′	12	0.24	3.8	15.83	II_B
peak III	18	0.52	2.6	5.00	III

Data represent the purification of RNA polymerases from 100 g of wheat germ.
*One unit of RNA polymerase activity is defined as the amount of enzyme catalysing the incorporation of 1 nmol GMP into acid-precipitable material in 20 min at 30°.

Table 2. Transcription of various DNA templates by wheat germ RNA polymerases II_A and II_B

DNA template	GMP incorporated (pmol)		
	II_A	II_B	II_B:II_A
Calf thymus, denatured	50.9	31.5	0.62
Wheat germ, denatured	56.4	106.0	1.88
Wheat germ, native	11.9	33.7	2.83

Standard RNA polymerase assay conditions were followed, except for the use of the indicated DNA templates (10 μg per 0.2-ml assay, in each experiment).

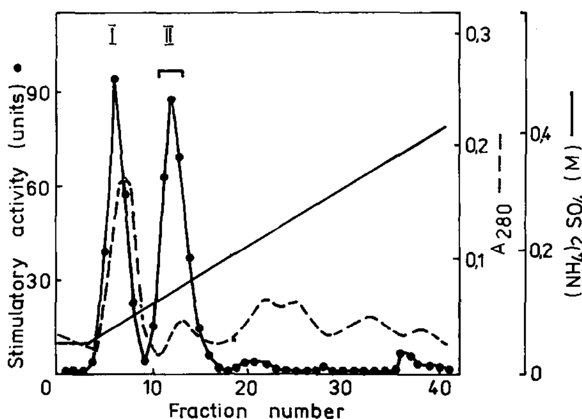

Fig. 3. Purification of RNA polymerase II stimulatory protein by chromatography on a DEAE-Sephadex A-25 column. Fraction NHCP-1e protein (see Table 3) was applied to the column (0.9 × 3 cm) in TEDGP and eluted with a linear gradient of ammonium sulphate from 0.05 to 0.5 M in 100 ml TEDGP. Fractions of 1.5 ml were collected and, in 10-μl aliquots, tested for the ability to stimulate RNA polymerase (a mixture of equal amounts of RNA polymerases II_A and II_B). A unit of the stimulatory activity was as defined in Table 3. Fractions corresponding to the stimulatory activity peak II were pooled (as indicated by the bracket) to obtain the NHCP-1eII preparation.

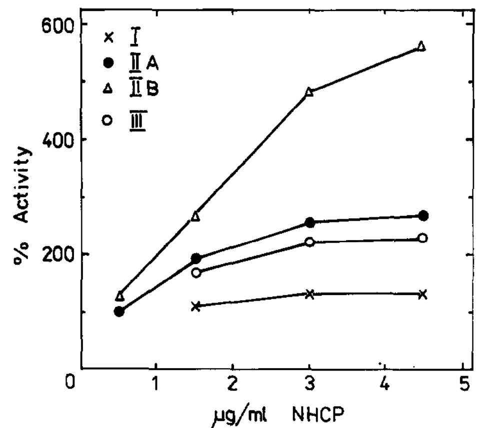

Fig. 4. Effect of NHCP-1eII on RNA polymerases I, II_A, II_B and III. The enzymes were assayed at various NHCP-1eII concentrations, as indicated. Activities of 100% (in the absence of the stimulatory NHCP) were similar to those given in Fig. 2 legend.

product length, although always larger than in its absence, depended upon both source and form of the template and was maximal when native wheat germ DNA was transcribed. This template was, therefore, chosen to illustrate the effect of the stimulatory protein on the RNA product size (Fig. 5). The NHCP-stimulated transcription resulted in the appearance of very long polyribonucleotide chains, including products larger than 23S rRNA, which were absent from the control (non-stimulated) transcription products. Evidently, the stimulation of [^{14}C]GTP incorporation was due to the increase of the product length, rather than to an increase in the number of initiation events.

In addition to the stimulation, NHCP-1eII was found to interfere with a chromosomal protein which inhibits RNA polymerase activity. As a result, the inhibition was reversed completely (Table 4). The inhibitory effect of other factors (α-amanitin, actinomycin D, RNase and DNase) could not, however, be reversed significantly.

The stimulatory protein NHCP-1eII was a specific phosphate acceptor of casein type protein kinase from wheat germ. The rate of its phosphorylation was three-

Table 3. Summary of purification of RNA polymerase II_B-specific stimulatory NHCP from wheat germ

Purification step	Fraction designation	Protein (mg)	Stimulatory activity (units*)	(units/mg)
1. 15 000 *g* pellet	Chromatin	280	nd	—
2. 0.2 M NaCl	Salt extract	15.3	nd	—
3. Bio-rex 70	NHCP-1	1.9	48	25
4. Heparin-Sepharose	NHCP-1e	0.5	76	152
5. DEAE-Sephadex	NHCP-1eII	0.1	333	3330

Chromatin was purified from a 100-g wheat germ sample according to ref. [11]. Steps 2–4 were made as described before [4]. For DEAE-Sephadex CC conditions (step 5), see Fig. 3. The stimulatory activity was tested in assays containing RNA polymerase II_B (2 μg) as the enzyme source (nd, non-detectable).

*One unit is defined as that amount of NHCP which, when added to the standard RNA polymerase assay, stimulates the enzyme activity by a factor of 2.5.

Table 4. Effect of NHCP-1eII on RNA polymerase II_B activity tested in the presence of various inhibitors

Inhibitor	Concn (μg/ml)	RNA polymerase activity (%) No NHCP-1eII	+ NHCP-1eII
None	—	100.0	295.0
NHCP-1b	0.3	21.4	106.2
α-amanitin	0.1	10.5	10.3
α-amanitin	1.0	1.7	1.9
Actinomycin D	5.0	23.7	26.8
RNase	50.0	40.0	43.6
DNase	50.0	14.5	16.3

Standard RNA polymerase assays were supplemented with the indicated inhibitors and incubated for 10 min either in the absence or in the presence of NHCP-1eII (2 μg/ml). The activity of 100% (neither inhibitor nor NHCP-1eII added) was 15 pmol GMP incorporated/assay.

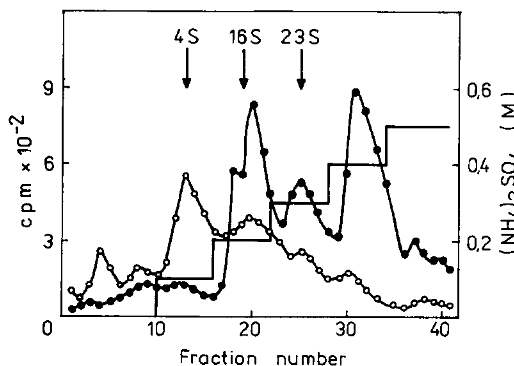

Fig. 5. Lysine-Sepharose CC of RNA products resulting from transcription of native wheat germ DNA by RNA polymerase II_B. A sample of transcripts, obtained either in the absence (○) or in the presence (●) of NHCP-1eII (2 μg/ml), was applied to a lysine-Sepharose 4B column in TMg and eluted with a series of salt steps (as indicated by the histogram; for details, see Experimental). Fractions of 1 ml were collected and assayed for TCA-precipitable radioactivity. The arrows refer to the positions of each of the RNA markers.

fold higher than that of casein itself (Table 5). Unexpectedly, however, the phosphorylated preparation was unable to stimulate RNA polymerases.

DISCUSSION

In this report, we have described the purification of RNA polymerase II_B, and a NHCP factor that stimulates its activity, from resting wheat embryos. The polymerase obtained prefers homologous to calf thymus DNA as template and actively transcribes native wheat germ DNA that allows us to postulate its direct participation in wheat genome transcription. The stimulatory NHCP does not co-purify with the enzyme and contains polypeptides which, with respect to their MWs (55 000 and 37 000), correspond to none of the known RNA polymerase II subunits [2, 3]. Instead, it resembles other loosely bound NHCP [6–8] and nucleoplasmic proteins [9] that are able to stimulate RNA polymerase II in assays containing naked DNA as template. In particular, the preparation is similar to that isolated previously from the same source [4] and may represent a somewhat better purified form of the same active component. It differs, however, from all thus far described stimulatory factors with respect to its specificity, being highly specific for form II_B of the class II enzyme.

Table 5. Phosphorylation of NHCP-1eII and other proteins by cyclic AMP-independent protein kinase from wheat germ

Substrate	^{32}P incorporated (cpm × 10^{-3}) −cAMP	+cAMP
NHCP-1eII	73.8	71.7
Bovine milk casein	24.1	25.0
Crude NHCP	20.6	21.3
Bovine serum albumin	6.3	6.5
Wheat gliadin	5.4	5.1
Calf histone, type II-A	1.9	2.2

All protein substrates were at a concentration of 10 μg per 0.1-ml kinase assay (see Experimental). Adenosine 3′:5′-cyclic monophosphate, when added, was 10^{-6} M. Crude NHCP was obtained according to ref. [11]. Background [^{32}P]ATP incorporation (2550 cpm/assay) was subtracted from all values.

The occurrence of the form II_B-specific RNA polymerase II stimulatory factor seems to indicate that the two structurally different enzyme forms, II_A and II_B, may differ from each other also with respect to their functions. For plant tissues, data have been reported to show that RNA polymerase II_A is a storage or precursor form of RNA polymerase II_B [3]. According to our observations, however, the increase in the level of RNA polymerase II_B activity in rapidly growing tissues may not necessarily be dependent on the conversion of form II_A into form II_B. A direct activation of the pre-existing II_B enzyme by its stimulatory factor seems to be more probable. A possibility of the conversion of polymerase II_A to polymerase II_B is also difficult to reconcile with the results of immunochemical studies which have shown that subunits IIa and IIb do not share the same antigenic element [10]. It seems, therefore, plausible to assume that the two enzyme forms may coexist within the same plant cell (independently of its metabolic status), but are regulated by two different mechanisms, one specific for form II_A and another for form II_B. The isolated protein factor (NHCP-1eII) seems to be an essential component of the latter mechanism.

EXPERIMENTAL

Materials. Wheat (*Triticum aestivum*) germ was obtained from a local mill. Chromatin was purified from the germ according to ref. [11]. Native wheat germ DNA was isolated by a conventional method [12] and purified additionally on a hydroxyapatite column [13]. Protein kinase was purified from wheat germ and the product obtained was similar to that described previously [14]. [γ-^{32}P]ATP was prepared by the method of ref. [15]. [8-^{14}C]GTP (Amersham), DEAE-Sephadex A-25 (Pharmacia) and all other reagents and materials were of commercial origin.

Isolation of total RNA polymerase activity. Initial steps (extraction, polymin P and $(NH_4)_2SO_4$ pptations) were as described in ref. [5]. A smaller amount (100 g) of wheat germ was, however, used and vols. of all the solns were scaled down correspondingly. The $(NH_4)_2SO_4$ fraction obtained (*ca* 120 mg) was dissolved in 15 ml of TEDGP and dialysed against the same buffer for 6 hr at 4°. To the dialysed soln, 10 g of DEAE-Sephadex A-25 (pre-equilibrated with, and suspended in, TEDGP) were added. The mixture was left for 1 hr at 0° with gentle stirring and then filtered through glass fibers. Non-adsorbed and loosely-bound products were removed from DEAE-Sephadex with TEDGP and TEDGP containing 0.1 M $(NH_4)_2SO_4$, respectively. To elute RNA polymerase activity, TEDGP + 0.5 M $(NH_4)_2SO_4$ was used. The extract obtained (*ca* 100 ml) was dialysed as above and served as the source of total RNA polymerase activity.

Isolation of RNA polymerase II stimulatory and inhibitory NHCP. Loosely-bound non-histone proteins were dissociated from wheat germ chromatin and fractionated to obtain stimulatory (NHCP-1e) and inhibitory (NHCP-1b) protein preparations as described in ref. [4]. The stimulatory protein was purified further by DEAE-Sephadex CC (see Fig. 3) prior to use.

Phosphorylation of the stimulatory NHCP. Kinase-catalysed phosphorylation of NHCP was measured under conditions similar to those described earlier [14]. The reaction mixture (0.1 ml) contained 50 mM Tris–HCl (pH 8), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.01 mM [γ-^{32}P]ATP (110 cpm/pmol), 10 μg NHCP, and 1 μg wheat germ protein kinase. After incubation for 10 min at 30°, TCA-pptable ^{32}P-radioactivity was measured as described in ref. [16]. For preparative purposes, a similar reaction was carried out, except that [^{32}P]ATP was replaced by unlabelled ATP and TCA-pptation was omitted.

RNA polymerase assay. The standard assay (0.2 ml) contained 50 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 0.25 mM each of ATP, CTP and UTP, 1 μCi [8-^{14}C]GTP (57 Ci/mol), 10 μg heat-denatured calf thymus DNA, and 2 μg of the enzyme. After incubation for 20 min at 30°, the reaction was stopped and TCA-pptable ^{14}C-radioactivity measured with a Packard liquid scintillation counter as described before [17].

Fractionation of RNA products. The transcription products were fractionated according to their size by lysine-Sepharose CC. A mixture of transcripts (obtained by 60 min incubation of standard RNA polymerase assay) was supplemented with RNA markers (tRNA, 16S rRNA and 23S rRNA, dissolved in TMg) and digested with RNase-free DNase (50 μg/ml) for 10 min at 30°. The sample was then cooled to 4° and applied to a lysine-Sepharose 4B column (0.8 × 5 cm). The column was washed (at 4°) first with 10 ml of TMg and then with five similar portions of TMg containing 0.1, 0.2, 0.3, 0.4 and 0.5 M $(NH_4)_2SO_4$, respectively. Fractions of 1 ml were collected and assayed for TCA-pptable ^{14}C-radioactivity. Positions of the internal RNA markers were found from spectrophotometric measurements.

SDS-polyacrylamide gel electrophoresis. Polypeptides in each of the purified protein preparations (RNA polymerases II_A and II_B, and NHCP-1eII) were resolved by slab gel electrophoresis [18] as described before [4].

Estimation of protein. This was made according to ref. [19] with bovine serum albumin as standard.

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