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ISOLATION OF AN RNA POLYMERASE II STIMULATORY PROTEIN FROM WHEAT GERM CHROMATIN

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Key Word Index—*Triticum aestivum*; *Secale cereale*; Gramineae; wheat embryo; transcription; non-histone chromosomal proteins; RNA polymerase II.

Abstract—Resting wheat embryos were found to contain an RNA polymerase II stimulatory factor. It occurred in the embryo cells as a loosely bound constituent of non-histone chromosomal proteins and was isolated as a non-dialysable, heat-labile preparation, containing a polypeptide with MW 37000 as the main component. The isolated preparation showed the stimulatory activity in a simple transcription assay, containing highly purified wheat germ RNA polymerase II as the enzyme source and either denatured or native DNA as template. No activity was found with *E. coli* RNA polymerase and RNA polymerase III was stimulated to a low extent. Similar stimulatory protein was isolated also from rye germ chromatin and found to be active with both rye and wheat class II RNA polymerases.

INTRODUCTION

Non-histone chromosomal proteins (NHCP) are generally believed to be involved in the regulation of gene expression in eukaryotic cells. In accordance with this view, various RNA polymerase stimulatory proteins have been found to be associated with chromatin in higher plant [1–3] and other [4–9] cell nuclei. With respect to their mode of action, the stimulatory NHCP seem to fall into two different groups. One group is represented by proteins which are able to stimulate transcription in simple assays, containing naked DNA as template and purified RNA polymerase preparations as the enzyme source [4, 5, 7, 8]. Thus, they may be expected to interact directly with DNA or polymerase. The other group of proteins can probably interfere or collaborate with other components of the nuclear transcriptional apparatus and will reveal their activity only in chromatin-templated transcription systems [1–3, 6, 9].

All RNA polymerase stimulatory NHCP thus far obtained from plant cells [1–3] clearly belong to the latter group. Wheat germ RNA polymerase II (EC 2.7.7.6) was shown, however, to be stimulated by a mammalian NHCP preparation also in simple assays, containing purified DNA as template [8]. We felt it probable, therefore, that in addition to the polymerase, resting wheat embryos may also contain its cognate stimulatory protein. In attempts to isolate such a protein, wheat germ chromatin was dissociated with sodium chloride solutions and the released NHCP fractions tested for the stimulatory activity. A loosely bound NHCP fraction showed the highest activity and that was partially purified and characterized.

RESULTS

When wheat germ chromatin was subjected to a step-wise dissociation (see Experimental), its template activity increased gradually (Table 1). The released NHCP fractions, however, did not inhibit the wheat germ RNA polymerase II activity. Instead, a considerable stimulation of the polymerase-catalysed [H]UTP incorporation was observed. The most loosely bound NHCP fraction, NHCP-1 (obtained with the use of 0.2 M sodium chloride), had the highest stimulatory effect. At its optimal concentration, which was found to correspond to 20 μg protein per standard assay (see Fig. 2), the NHCP-1 fraction stimulated incorporation of [^{3}H]UTP by a factor of 2.5. The NHCP-1 fraction itself had no RNA polymerase activity, did not stimulate [^{3}H]UTP incorporation in the absence of other ribonucleoside triphosphates or DNA and was unable to reverse the inhibitory effect of α-amanitin (Table 2). It lost the stimulatory activity after heating for 2 min at 100°.

The RNA polymerase-catalysed reaction product, formed either in the presence or absence of NHCP-1, remained acid-insoluble after DNA-ase and Pronase treatments but was readily hydrolysed by 0.3 M potassium hydroxide. The ratio of radioactive UMP to uridine in the alkaline hydrolysate was, however, considerably higher for the NHCP-stimulated reaction product (726 vs 450) indicating that the average length of polyribonucleotide chains formed by RNA polymerase could be larger when NHCP-1 was present in the assay. The observed increase could not result from the inhibition of RNA-ase activity since the highly purified RNA polymerase preparation used as the enzyme source was free of such activity.

Table 1. Step-wise dissociation of wheat germ chromatin

Dissociation step	Protein fraction obtained: Designation	Yield (mg)	Activity (% stimulation)	Chromatin template activity (%)
No treatment	—	—	—	100
0.2 M NaCl	NHCP-1	1.6	154	94
0.3 M NaCl	NHCP-2	2.0	70	133
0.5 M NaCl	NHCP-3	5.2	13	207
0.8 M NaCl	NHCP-4	1.1	139	788

Chromatin of 100 g wheat germ was dissociated successively with the indicated NaCl solutions. NHCP obtained on each of the dissociation steps was added (20 μg) to standard the RNA polymerase assay which, in the absence of NHCP, incorporated 7×10^4 cpm [^{3}H]UTP. Stimulation is expressed as percentage increase in the [^{3}H]UTP incorporation over this control value. Changes in chromatin template activity were also monitored and are expressed as a percentage of a value (4440 cpm) found with untreated chromatin.

Table 2. Requirements for the NHCP-1 fraction stimulatory activity

Test conditions	[^{3}H]UTP incorporated (cpm $\times 10^{-3}$): In the absence of NHCP-1	In the presence* of NHCP-1
Standard RNA polymerase assay	69.7	177.0
− RNA polymerase	< 0.1	< 0.1
− ATP, CTP and GTP	1.2	1.3
− DNA	< 0.1	< 0.1
+ α-Amanitin (0.1 μg)	1.8	1.5
+ Bovine serum albumin (20 μg)	70.3	—

*20 μg per 0.1-ml assay.

All the above described observations made us believe that the NHCP-1 fraction may contain a component which specifically stimulates RNA polymerase activity and attempts to purify this component were undertaken. When the NHCP-1 fraction was chromatographed on a heparin–Sepharose column, partial separation of several UV-absorbing components was achieved (Fig. 1). They influenced RNA polymerase II activity in a different manner. Products eluted at low salt concentrations (peaks a–d) were inhibitory, whereas those eluted at higher salt concentrations (peaks e–g) stimulated the enzyme. The highest stimulatory activity showed a product eluted with *ca* 0.35 M ammonium sulphate (peak e). The column fractions corresponding to the UV-absorbing peak, e, were therefore pooled and were lyophilized after dialysis. The NHCP-1e preparation obtained stimulated RNA polymerase more efficiently than did NHCP-1, from which it originated. This time, the 2.5-fold stimulation was achieved with the use of 4 μg protein per standard assay (0.1 ml). The stimulatory effect remained, however, within this range even if much higher concentrations of NHCP-1e were used (Fig. 2) and was independent of the incubation time (Fig. 3).

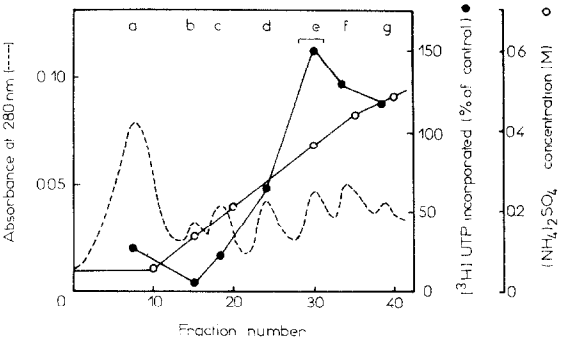

Fig. 1. Fractionation of loosely bound NHCP on a heparin–Sepharose column. The NHCP-1 fraction (2.2 mg) of wheat germ was applied to the column (5 × 0.9 cm) and eluted with a 100-ml ammonium sulphate gradient (0.05–0.55 M in 50 mM Tris–HCl, pH 8.0). Fractions of 2 ml were collected and, in 20 μl portions, tested for their effects on RNA polymerase-catalysed [^{3}H]UTP incorporation. The results are expressed as a percentage of the control incorporation (7×10^4 cpm). Fractions corresponding to peak e were pooled (as indicated by the bracket) to obtain the NHCP-1e preparation.

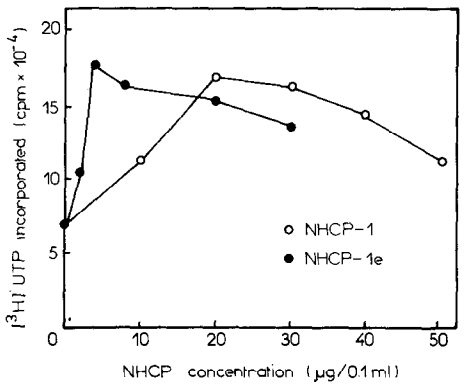

Fig. 2. Effects of NHCP concentration on RNA polymerase-catalysed incorporation of [^{3}H]UTP. Standard assay conditions were followed except for the addition of the NHCP-1 or NHCP-1e preparations, as indicated.

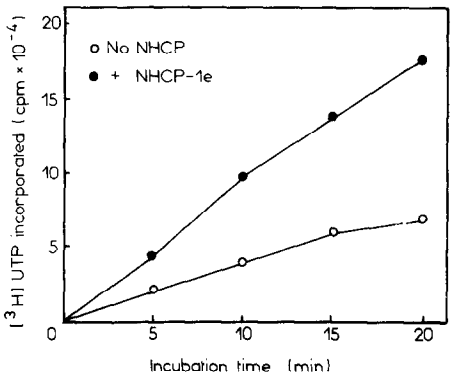

Fig. 3. Rates of RNA polymerase-catalysed incorporation of [^{3}H]UTP in the absence and in the presence of NHCP-1e. Standard RNA polymerase assays and the assays supplemented with 4 μg of the NHCP-1e preparation were incubated for the indicated time periods.

Electrophoretic analyses indicated that the isolated NHCP-1e preparation contained a polypeptide of MW 37 000 as the main component and two minor components (with MW 66 000 and 28 000, respectively). With regard to the MW neither of them correspond to any of the known RNA polymerase II subunits. The minor components could not be removed either by phosphocellulose or by DEAE-cellulose CC (performed according to ref. [10]) and further experiments were carried out with the NHCP-1e preparations as the source of the stimulatory factor. The preparation was free of nucleic acid contamination and showed neither nuclease nor terminal nucleotidyltransferase activity. Similarly, tests for protein kinase activity were negative.

In attempts to elucidate whether the stimulatory NHCP-1e was specific for any of the transcriptional system components, its activity was tested with various DNA templates and RNA polymerases. The results obtained are summarized in Table 3. The activity was found to be independent of the DNA source and responded only slightly to the template form (somewhat lower stimulation was observed when non-denatured DNA was used instead of the heat-denatured one). Likewise, pre-incubation of DNA with the NHCP-1e preparation had no effect, indicating that the active component does not introduce any change in the template that would then influence the transcription rate. Within the two plant species compared (wheat and rye), the stimulation depended upon neither the enzyme nor the factor source. The extent of stimulation depended, however, upon the class of RNA polymerase used. While RNA polymerase II was stimulated efficiently, the class III enzyme was stimulated only slightly (less than 1.3-fold) and no stimulation was found for the prokaryotic RNA polymerase-catalysed reaction. Thus, the stimulatory effect of the NHCP-1e preparation seems to be specific for the enzyme, rather than for the template.

Table 3. Effects of NHCP-1e on RNA synthesis in various transcription assays

RNA polymerase		DNA template		[^{3}H]UTP incorporated (cpm × 10^{-3})		
Source	Class	Source	Form	No NHCP added	+ Wheat germ NHCP-1e	+ Rye germ NHCP-1e
Wheat germ	II	Calf thymus	Denatured	70.3	181.7	169.0
Wheat germ	II	Calf thymus	Native	42.9	76.7	70.4
Wheat germ	II	Calf thymus	Pre-incubated*	69.4	172.1	n.d.
Wheat germ	II	Wheat germ	Denatured	77.1	193.2	190.0
Wheat germ	II	Rye germ	Denatured	72.2	189.0	194.6
Wheat germ	III	Calf thymus	Denatured	12.0	15.1	n.d.
Rye germ	II	Calf thymus	Denatured	69.5	158.6	166.5
Rye germ	III	Calf thymus	Denatured	14.7	n.d.	18.0
E. coli		Calf thymus	Denatured	51.3	39.4	41.3

The isolated NHCP-1e preparations were added (4 μg) to transcriptional test systems containing the indicated enzyme and template and all other components of the standard RNA polymerase assay.

n.d.: Not determined.

*Incubated with NHCP-1e and heat-denatured prior to use.

DISCUSSION

With respect to its ability to stimulate wheat germ RNA polymerase II, the NHCP preparation resembles that isolated by Legraverend and Glazer [8] from rat liver nuclei. Both plant and mammalian NHCP preparations stimulated the enzyme activity under simple assay conditions (with the use of deproteinized DNA as template) and at their optimal concentrations (which were also roughly the same) increased the reaction rate to a similar extent. Likewise, neither of them stimulated *E. coli* RNA polymerase. Although Legraverend and Glazer's NHCP preparation contained a polypeptide of MW 66 000 as the main component, the polypeptide of a similar MW occurred in our preparation only as a contaminant accompanying the main polypeptide of MW 37 000. RNA polymerase II stimulatory proteins of the same MW (37 000) have been recently isolated by Sawadogo *et al.* [11] from yeast cells, and by Sasaki and collaborators [2, 3] from germinating wheat embryos. However, neither of them seems to correspond to our preparation. The yeast factor was shown to be associated with the RNA polymerase itself (see also ref. [12]), rather than with the chromatin. Sasaki's factor, on the other hand, was a typical NHCP but was absent from resting embryos. It appeared at a specific germination stage and influenced the specificity rather than the rate of transcription. These differences make us believe that the NHCP preparation isolated from resting embryos represents a new example of RNA polymerase II stimulatory factors, belonging to a group of NHCP which are able to stimulate the enzyme activity in simple assays. Its possible relation to the recently described class II gene-specific transcription factors [10, 13] remains to be elucidated.

EXPERIMENTAL

Materials. Wheat (*Triticum aestivum*) and rye (*Secale cereale*) germs were obtained from a local mill. RNA polymerase II was isolated from the dry embryos essentially according to ref. [14] as described previously [15]. RNA polymerase III was similar to that described earlier [16]. All other biochemicals and reagents, including *E. coli* RNA polymerase (Boehringer), [5-^{3}H]UTP (Amersham) and heparin–Sepharose 6B (Pharmacia) were of commercial orgin.

Preparation of chromatin. Chromatin was isolated and purified from dry wheat (or rye) embryos according to the method of ref. [17]. *Ca* 250 mg chromatin (DNA wt basis) were obtained from a 100 g embryo sample.

Step-wise dissociation of chromatin and isolation of stimulatory NHCP preparations. The purified chromatin preparation (250 mg) was suspended in 200 ml of a 0.2 M NaCl soln containing 10 mM Tris–HCl (pH 8), 15 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulphonyl fluoride. The suspension was left for 4 hr at 4° with gentle stirring. Products released under these dissociation conditions were separated from the chromatin residue by 15 min centrifugation at 15 000 g. The supernatant (fraction 1) was saved and the chromatin residue (pellet) was again resuspended and dissociated under similar conditions except that this time 0.3 M NaCl was used (fraction 2 was obtained). The stepwise dissociation was continued further using 0.5 M (fraction 3) and finally 0.8 M (fraction 4) NaCl solns. Fractions 1–4 were then subjected to a PEG–dextran partition procedure followed by Bio-Rex 70 CC [17] to obtain the NHCP-1, NHCP-2, NHCP-3 and NHCP-4 preparations, respectively. Each of them was dialysed against 2 mM Tris–HCl (pH 6.8), 2 mM mercaptoethanol and lyophilized.

The NHCP-1 preparation was fractionated further by chromatography on a heparin–Sepharose 6B column under conditions similar to those described in ref. [18] with experimental details as given in the legend to Fig. 1. The column effluents were monitored for protein by determination of the *A* 280 nm and the salt concns were determined by conductivity measurements.

Transcription assay. The standard RNA polymerase assay (0.1 ml) contained 100 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM each of ATP, CTP, and GTP, 1 μCi of [5-^{3}H]UTP (11 Ci/mmol), 20 μg heat-denatured calf thymus DNA, and 1 μg wheat germ RNA polymerase II. After incubation for 20 min at 30°, the TCA-precipitable radioactivity was measured [15]. Changes from the standard assay conditions are specified in the Results.

To hydrolyse the radioactive reaction product, *ca* 100 μg yeast tRNA were added to the incubation mixture prior to the pptn. The ppt was pre-treated with pancreatic RNA-ase-free DNA-ase (5 μg/ml, 37°, 15 min) and Pronase (0.1 mg/ml, 30°, 30 min) and then hydrolysed with 0.3 M KOH at 37° for 15 hr. The resulting degradation products were separated as described previously [19].

Chromatin template activity was measured under conditions of the standard RNA polymerase assay, except that DNA was replaced by an equivalent amount of the chromatin preparation.

Tests for interfering enzyme activities. Nuclease activity was measured as described earlier [20] using DNA and RNA as substrates. Terminal nucleotidyltransferase activity was assayed according to ref. [21] with the use of [^{3}H] UTP as the nucleotide residue donor. Assays for protein kinase activity were similar to those described previously [15] and contained either wheat germ RNA polymerase II or casein as the expected P_1 group acceptors.

SDS–polyacrylamide slab gel electrophoresis. Gels were run using the discontinuous system described in ref. [22] with a 5% stacking gel and a 10% running gel. The analysed preparations (10–70 μg protein) were dissolved in 63 mM Tris–HCl (pH 6.8), 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS and heated at 90° for 2 min prior to application. Commercially available proteins of known MW (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean tripsin inhibitor, and lactalbumin) were used as standards. Gels were stained with Coomassie Brilliant Blue and destained in HOAc.

Determination of nucleic acid content. A sample of the analysed preparation (up to 0.5 mg of protein) was hydrolysed with conc. $HClO_4$ and the hydrolysate was assayed for the presence of purine and pyrimidine bases by PC in H_2O-satd *n*-BuOH as solvent system [23].

Estimation of protein. Protein was estimated by the method of ref. [24], using bovine serum albumin as standard.

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