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Purified phytochrome influences *in vitro* transcription in rye nuclei

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Nuclei isolated from dark-grown seedlings of rye by Percoll density gradient centrifugation incorporate radioactive UTP into RNA. Transcription is reduced to ~50% by the addition of α-amanitin (5 μg/ml) and correspondingly a decrease of the label in the mRNA fraction is seen on agarose gels. Purified 124-kd phytochrome in its far-red absorbing form (P_{fr}) increases the incorporation of labeled UTP by 40 – 70% above untreated controls, whereas pre-illumination with far-red light or addition of α-amanitin prevents this effect. Nuclei isolated from light-grown seedlings show only an increase of ~5% upon addition of P_{fr}. Other proteins such as bovine serum albumin or cytochrome c do not enhance the rate of transcription. We conclude that the accumulation of mRNA species is influenced by phytochrome in its P_{fr} form.

Key words: nuclei/phytochrome/rye/transcription

Introduction

For higher plants, light is not only an energy source but also the signal for induction of photomorphogenesis. Exposure of dark-grown seedlings to light is accompanied by changes of many physiological and enzyme activities (Mohr and Schopfer, 1978). In recent years the influence of light on transcription became obvious when, in several plant species, changes of mRNA levels were observed upon red light illumination (Apel, 1979, 1981; Sasaki *et al.*, 1981, 1983; Tobin, 1981; Cuming and Bennett, 1981; Gallagher and Ellis, 1982; Gottmann and Schäfer, 1982; Gollmer and Apel, 1983; Colbert *et al.*, 1983; Stiekama *et al.*, 1983; Thompson *et al.*, 1983; Lamb and Lawton, 1983; Silverthorne and Tobin, 1984). The net increase in the rate of transcription is mainly due to the synthesis of mRNA sequences coding for components of the chloroplast, e.g., the light-harvesting a/b protein or the ribulose-1,5-bisphosphate carboxylase. This red light photocontrol of gene expression is exerted *via* phytochrome, a protein which contains a linear tetrapyrrole chromophore (Mohr, 1966; Rüdiger, 1980). The receptor exists in two photo-interconvertible forms: a red absorbing (P_r) and a far-red absorbing (P_{fr}) form. The physiological behaviour of plants indicates that P_{fr} is the active form and P_r the inactive form (Mohr and Schopfer, 1978; Pratt, 1979). Up until 1982 the 114/118-kd species of phytochrome was considered to represent the undegraded photoreceptor. However, recently a 124-kd monomer was shown to represent the native photoreceptor in rye and oat (Vierstra and Quail, 1982; Kerscher, 1983; Litts *et al.*, 1983). This opened up the possibility of studying the photocontrol of gene expression *in vitro* and in the absence of light. Here, we report that isolated phytochrome in its active P_{fr} form enhances mRNA synthesis in isolated nuclei.

Results and Discussion

The isolation of native phytochrome molecules is easier to accomplish when they are in the P_{fr} state, which is more resistant to proteolysis than the P_r form (Kerscher, 1983). The best preparation so far obtained from rye, however, still contained a 50-kd impurity (Figure 1, lane 1) which can either be removed by sucrose density gradient centrifugation (Figure 1, lane 3) or immunoaffinity chromatography (Figure 1, lane 2). Phytochrome P_r is produced from purified P_{fr} by illumination with light of λ_{max} 730 nm. The spectra of both species are compared in Figure 2. The 114/118-kd phytochrome is obtained by the same purification procedure as described for P_{fr}, but without prior transformation into P_{fr}.

We used nuclei from dark-grown (etiolated) rye seedlings in our experiments and nuclei from light-grown seedlings as controls. Etiolated seedlings contain high levels of phytochrome in its inactive P_r form and light induces increased mRNA synthesis in their nuclei (Gallagher and Ellis, 1982; Silverthorne and Tobin, 1984; Mösinger and Schäfer, 1984). In contrast, light-grown seedlings contain a mixture of P_r and P_{fr} in a photoequilibrium at a very low level (Lamb and

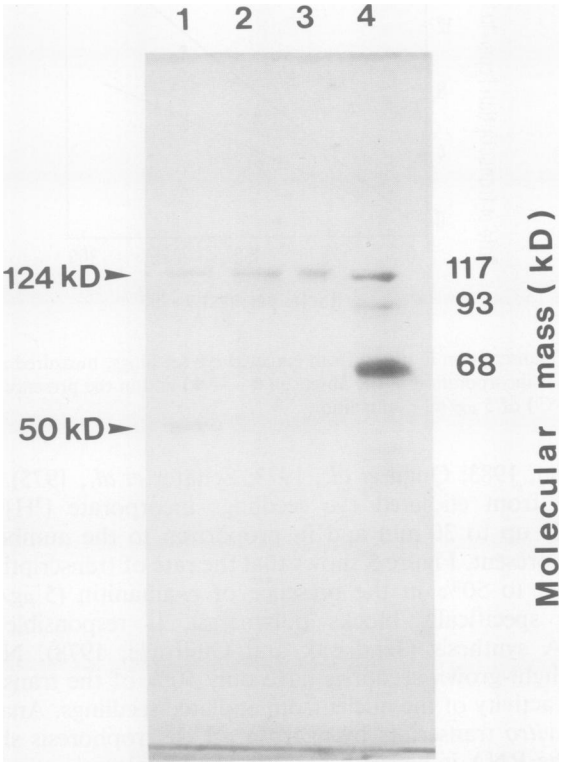

Fig. 1. SDS-PAGE (5 – 20% acrylamide linear gradient) of purified 124-kd phytochrome. **Lane 1**: after ACA-34 Ultrogel filtration; **lane 2**: after immunoaffinity chromatography; **lane 3**: after sucrose density gradient centrifugation; **lane 4**: protein mol. wt. markers.

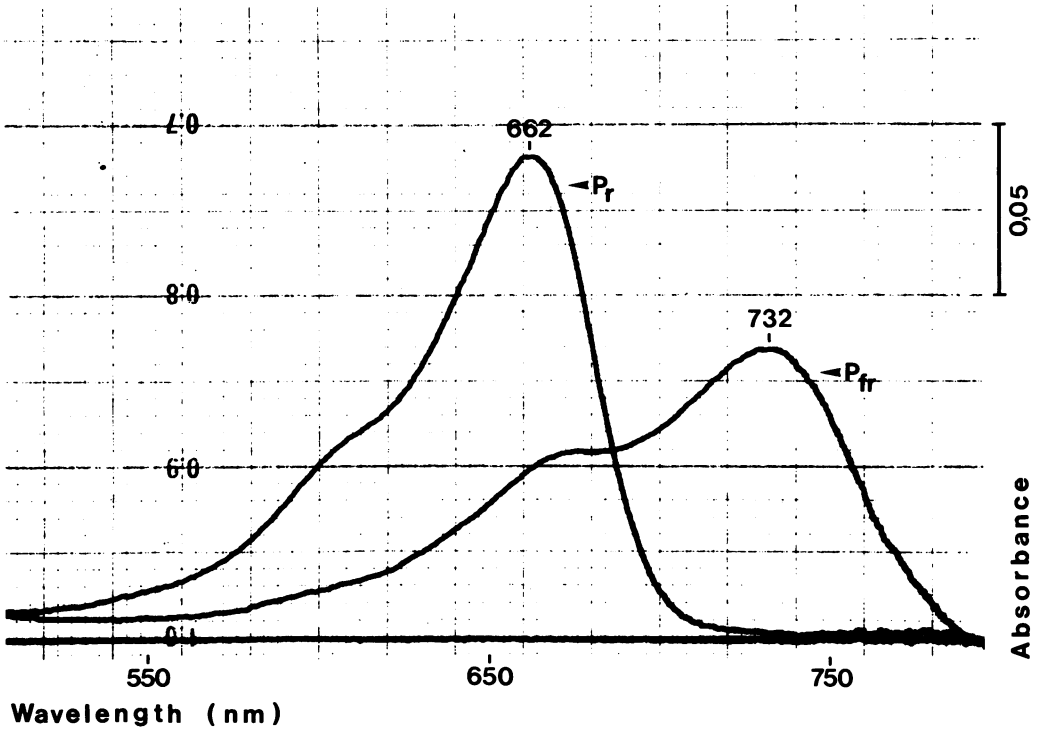

Fig. 2. Spectra of native phytochrome before (P_{fr}, λ_{max} 732 nm) and after (P_r, λ_{max} 662 nm) far-red light illumination.

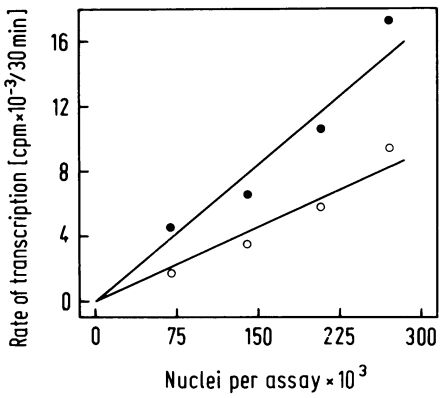

Fig. 3 Transcription in nuclei from etiolated rye seedlings, measured as [^{3}H]UTP incorporation in the absence (●——●) and in the presence (○——○) of 5 μg/ml α-amanitin.

Lawton, 1983; Quail *et al.*, 1973; Schäfer *et al.*, 1975). The nuclei from etiolated rye seedlings incorporate [^{3}H]UTP linearly up to 30 min and in proportion to the number of nuclei present. Figure 3 shows that the rate of transcription is reduced to 50% in the presence of α-amanitin (5 μg/ml), which specifically blocks polymerase II responsible for mRNA synthesis (Jendrisak and Guilfoyle, 1978). Nuclei from light-grown seedlings have only 50% of the transcriptional activity of the nuclei from etiolated seedlings. Analysis of *in vitro* transcripts by agarose gel electrophoresis shows that the RNA is heterodisperse in size ranging up to ~23S (Figure 4, lane 1). If α-amanitin is added to the incubation mixture, the size distribution of the transcripts is unaltered, but the level of incorporation decreases (Figure 4, lane 2). No addition of ribonuclease inhibitor also resulted in a heterodisperse RNA, but of smaller size, up to ~20S (results not shown).

Figure 5 presents an experiment processed in duplicate, demonstrating enhancement of transcription by phytochrome P_{fr} in nuclei from etiolated seedlings. Nuclei from light-grown seedlings show only a slight increase in their rate of transcription. Interestingly the 114/118-kd phytochrome in its P_{fr} form only increases the rate by 8% although twice the amount of the native phytochrome was added. Transcription is maximally enhanced by 70% at a level of ~1 μg phytochrome per 200 000 nuclei. The concentration of phytochrome in etiolated seedlings of oat was measured spectroscopically and immunologically and was found to be ~10 μg P_r/g tissue (Shimazuki *et al.*, 1983) corresponding to 0.3 μg per 200 000 nuclei, used in our assay system. Therefore our incubation conditions and the observed effects on transcription are comparable with the *in vivo* situation. It should be mentioned that compartmentation and vacuolization increases the effective concentration of phytochrome *in vivo* but not *in vitro*.

In a second series of experiments we compared the effectiveness of P_{fr} and P_r and other proteins (Figure 6). The results with P_{fr} confirm those from Figure 5; however, the average of the six experiments processed in duplicate (lane 1–4) shows maximal activation of 46% at 4 μg added P_{fr}. When P_r was produced from P_{fr} by irradiation with far-red light (see spectrum in Figure 2) prior to the addition to the nuclei, only a small but measurable increase in transcriptional activity occurs. We cannot explain this small effect of P_r, which is thought to be inactive, but point out that the nuclei are prepared in 0.5% Triton X-100 and therefore presumably have different permeability properties compared with nuclei *in vivo*. Studies on the interaction of nuclei isolated without detergent with P_{fr} and P_r are now in progress. The increase in rate of transcription by P_{fr} is inhibited by α-amanitin (5 μg/ml) as shown by lanes 7–9 of Figure 6, which clearly demonstrates that the P_{fr}-stimulated increase is due to mRNA synthesis. Proteins such as bovine serum albumin and cyto-

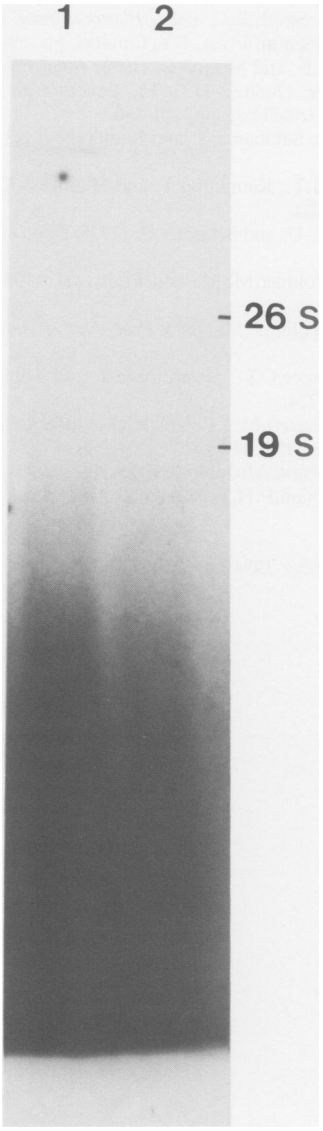

Fig. 4. Autoradiograms showing the size distribution of RNA synthesized by nuclei isolated from rye shoots (**lane 1**). Incubation for **lane 2** also contained 5 μg/ml α-amanitin. RNA transcripts labeled with [α-^{32}P]UTP were purified and fractionated on a formaldehyde agarose gel. For drying, the gel was cut at the lower end of the 4S region. The marker molecules were 26S and 19S rRNA from *Physarum* and were stained with ethidium bromide.

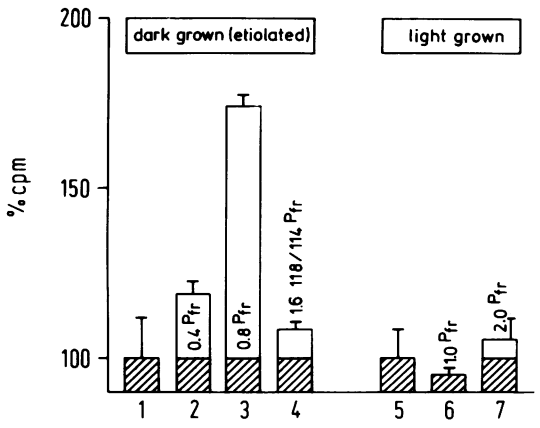

Fig. 5. Increased [^{3}H]UTP incorporation into RNA induced by native phytochrome. Values are expressed as percentage of control (11 000 c.p.m.), **lane 1. Lanes 2** and **3** represent the addition of 0.4 and 0.8 μg P_{fr}, respectively, **lane 4** that of 1.6 μg 114/118-kd P_{fr}. **Lanes 5–7** present results with nuclei from light-grown seedlings, 100% are 5500 c.p.m. (**5**: no addition, **6**: 1 μg native P_{fr} and **7**: 2 μg native P_{fr}). All samples contain 200 000 nuclei in 60 μl incubation mixture and are kept at 25°C for 30 min. Vertical bars represent the average of two parallel experiments.

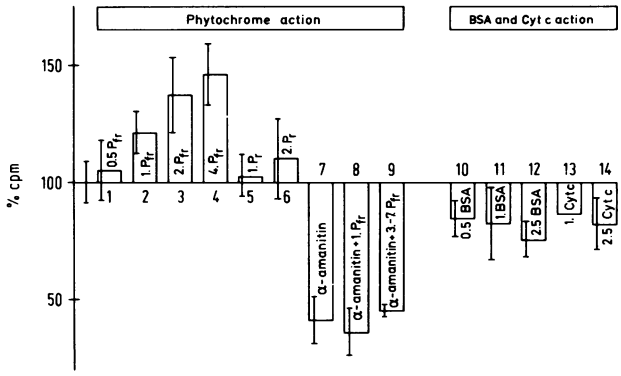

Fig. 6. Comparison of native phytochrome P_{fr}, P_r and two non-photoreceptor proteins, bovine serum abumin (BSA) and cytochrome c (cyt c). Additions are in μg and the values are expressed as percentage of control (11 000 c.p.m.). Vertical bars represent the SD of six experiments, processed in duplicate.

chrome c cannot stimulate [^{3}H]UTP incorporation into RNA, but rather seem to be slightly inhibitory (lanes 10–14, Figure 6). This strengthens the idea of a unique role of P_{fr}. Presently we are isolating the gene for the small subunit of ribulose-1,5-bisphosphate carboxylase as a probe in order to demonstrate specific induction of gene expression by P_{fr}. Furthermore our intention is to find out whether P_{fr} is interacting directly with chromatin or a second messenger is involved in gene expression.

In conclusion, we believe that this first demonstration of mRNA accumulation induced by purified P_{fr} in the absence of light, and in an *in vitro* system, will be a suitable basis for biochemical research on the molecular details of photomorphogenesis in plants.

Materials and methods

Diaflo YM5 membranes were from Amicon (Witten, FRG), Nylon filters from Eckert and Franz (Waldkirch, FRG), Vermiculite nr.4 from Specht (Munich, FRG), Eppendorf tubes from Netheler and Hinz (Hamburg, FRG), Waring blendor from Waring Commercial Division (New Hartford, USA), 40-W Fluora fluorescent lamps from Osram (Munich, FRG), ATP, CTP, GTP, UTP, α-amanitin and dextran 40 from Sigma (Munich, FRG), Ultrogel ACA 34 and HA-Ultrogel from LKB (Gräfelfing, FRG), Sephadex G-50, Percoll, Ficoll 400 and BrCN-Sepharose from Pharmacia (Freiburg, FRG), cellulose DE52 and GF/C filters from Whatman (Maidstone, UK), morpholinopropane sulfonic acid from Serva (Heidelberg, FRG), Seaken HE agarose from Johnson & Co (Erkrath, FRG) Rotiszint 22 from Roth (Karlsruhe, FRG). All other chemicals were from Merck (Darmstadt, FRG).

Purification of phytochrome

All operations are carried out under green safelights at 4°C, according to Kerscher (1983). All buffers contained 5 mM 2-mercaptoethanol. 1.2–1.4 kg of rye seedlings (*Secale cerale* cv. Danko; BayWa AG, Argelsried, FRG) grown in the dark for 3 days at 27°C were irradiated with 40-W Fluora lamps for 2.5 min (4800 lx) and then homogenized with 1 litre of ice-cold 200 mM Tris-HCl pH 8.0 containing 2.5 mM Na_2-EDTA in a one-gallon Waring blendor. After filtration through a Nylon cloth (280 μm) the filtrate was centrifuged at 54 000 *g* for 1 h. The supernatant was mixed with 0.5 volumes of a saturated $(NH_4)_2SO_4$ solution adjusted to pH 7.8 with Tris-base and centrifuged at 27 000 *g* for 1 h. The pellet was resuspended in 100 ml of 100 mM KPO_4 pH 7.8, centrifuged at 200 000 *g* for 1 h, desalted on a Sephadex G-50 column (5 x 18 cm) and chromatographed on Whatman DE52 cellulose (2.6 x 50 cm) using a linear gradient (2 x 600 ml) from 50 to 250 mM KCl in 50 mM Tris-HCl pH 7.3. The phytochrome-containing fractions were pooled, purified on hydroxyapatite-Ultrogel (1.6 x 12 cm) using a 2 x 100 ml linear gradient of 2–40 mM KPO_4 pH 7.8 in 100 mM KCl. Fractions containing

phytochrome were concentrated by ultrafiltration in an Amicon cell equipped with a YM5 membrane. After gel filtration on Ultrogel AcA-34 (1.6 x 85 cm) phytochrome was again concentrated by ultrafiltration, layered onto a sucrose gradient (10–30%, w/v) and centrifuged at 200 000 *g* for 24 h. Phytochrome-containing fractions were pooled and stored at −70°C until use. The purity of the preparations was analyzed by SDS gel-electrophoresis using a linear gradient from 5–20% (Laemmli, 1970).

When using an immunoaffinity column, phytochrome containing fractions were applied to the column which was washed with the same buffer. The pooled phytochrome-containing fractions were concentrated by ultrafiltration. The immunoaffinity column was prepared by coupling purified antisera, raised against the 50-kd impurity, to BrCN-Sepharose, using conventional methods (Livingstone, 1974).

Light treatment

Phytochrome was phototransformed from P_{fr} to P_r by irradiating the sample at 4°C for 15 min. Far-red light was provided by a projector with a 730 nm (6 nm band width) interference filter (Schott, Mainz, FRG).

Isolation of nuclei and transcription

The isolation of nuclei was according to Gallagher and Ellis (1982) except that the rye seedlings were homogenized in a Waring blendor (low speed, 10 s) and the homogenate filtered successively through three pieces of Nylon cloth (280 μm, 130 μm, 20 μm). The nuclei are kept frozen in Eppendorf tubes (200 000 nuclei/tube). The standard transcription assay (60 μl) contains 200 000 nuclei in 50 mM Tris-HCl pH 8.5, 5 mM $MgCl_2$, 2.5 mM dithioerythritol and 12.5% glycerol (v/v). Phytochrome, other proteins and α-amanitin are added before transcription is started and the mixture kept at 4°C for 5 min. Transcription is started by the addition of $(NH_4)_2SO_4$ (50 mM), $MnCl_2$ (2 mM), ATP, GTP, CTP (each 0.5 mM), and [^{3}H]UTP (0.185 MBq; 1.59 TBq/mmol; Amersham Buchler, Braunschweig, FRG) and the assay mixture incubated at 25°C for 30 min. Transcription is terminated by the addition of 200 μl 6 mM UTP in 200 mM $Na_4P_2O_7$ followed by 1 ml of 10% trichloroacetic acid (TCA) (w/v). The mixture is transferred on to Whatman GF/C filters, which are washed with 4 ml of 5% TCA (w/v) in 50 mM $Na_4P_2O_7$, 4 ml of 5% TCA (w/v) in 500 mM KCl and twice with 4 ml of 5% TCA (w/v) in 50 mM $Na_4P_2O_7$ over a period of 15 min and then washed once with ethanol (Mösinger and Schäfer, 1984). The filters are transferred to 5 ml Rotiszint 22 and counted by liquid scintillation spectrometry with an efficiency of 30% for ^{3}H. Preparative RNA synthesis was carried out in 300 μl with 1–1.5 x 10^6 nuclei under the conditions described above. Transcription was performed in the presence of 0.37–1.11 MBq [α-^{32}P]UTP (15.17 TBq/mmol; Amersham Buchler, Braunschweig, FRG) and 5.5 μl ribonuclease inhibitor (BRL, Neu Isenburg, FRG). The isolation of RNA was based on the method of Silverthorne and Tobin (1984) and final samples were fractionated in a 1.5% agarose gel in 20 mM morpholinopropane sulfonic acid, pH 7.0/5 mM sodium acetate/1 mM EDTA/formaldehyde (6%). For drying the gel was cut at the lower end of the 4S region and then exposed to X-ray film (Trimax, 3M Deutschland, Neuss, FRG).

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