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Phytochrome: molecular properties and biogenesis

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Native *Avena* phytochrome, recently shown to have a monomeric molecular mass of 124 kDa, has molecular properties that differ significantly from those of the extensively characterized '120' kDa or 'large' phytochrome preparations now known to contain a mixture of proteolytically degraded 118 and 114 kDa polypeptides. For example, 124 kDa phytochrome has a blocked N-terminus, a P_{fr} λ_{max} of 730 nm, a higher photostationary state in red light (86% P_{fr}), exhibits no dark reversion and shows no differential reactivity of P_r and P_{fr} toward a chemical probe of hydrophobic domains. The data indicate that the proteolytically removed 6–10 kDa polypeptide segment(s) is critical to the spectral and structural integrity of the photoreceptor; that at least part of the cleaved domain is located at the N-terminus of the molecule; that this domain influences the chemical reactivity of the chromophore with the external medium; and that a current hypothesis that P_r–P_{fr} photoconversion results in the exposure of a hydrophobic domain on the molecule is inconsistent with the properties of native phytochrome.

Phytochrome has been found to exert rapid negative feedback control over the level of its own translatable mRNA. P_{fr} formation in etiolated tissue causes a decline in translatable phytochrome mRNA that is detectable within 15–30 min and that results in more than a 95% reduction within 2 h. Less than 1% P_{fr} is sufficient to induce 60% of the maximum response, which is saturated at 20% P_{fr} or less. The rapidity of this autoregulatory control makes phytochrome itself an attractive system for investigating phytochrome-regulated gene expression.

A project to clone phytochrome complementary DNA (cDNA) has been initiated. A major obstacle in this work has been the unexpectedly low abundance of phytochrome mRNA, less than 0.005% of the poly(A) RNA in etiolated tissue. cDNA made from poly(A) RNA enriched *ca.* 200-fold in phytochrome mRNA has been cloned and bacterial colonies have been screened with a synthetic oligodeoxynucleotide hybridization probe. The sequence of this probe was derived from a known partial amino acid sequence of the phytochrome protein. Difficulties encountered with this approach are discussed.

Introduction

We have been investigating the molecular properties and biogenesis of phytochrome for the ultimate purpose of understanding the mechanism by which the photoreceptor regulates plant development. This paper describes three facets of the investigation.

The phytochrome molecule

Efforts to elucidate the mechanism of phytochrome action have focused considerable attention on the physicochemical properties of the chromoprotein and on the molecular differences between P_r and P_{fr} (see Pratt 1982). The success of this approach clearly depends on the isolation of phytochrome in an undegraded, undenatured form. Initial attempts in the mid-1960s to purify the photoreceptor yielded a photoreversible chromoprotein with an apparent molecular mass of 60 kDa (Mumford & Jenner 1966), and a considerable amount of

information on its properties was assembled (Briggs & Rice 1972; Pratt 1979, 1982). In the early 1970s, however, Briggs and coworkers isolated a molecule with a monomer size of about 120 kDa and demonstrated that the 60 kDa species was derived proteolytically from the larger polypeptide during purification (Gardner *et al.* 1971; Rice & Briggs 1973; Rice *et al.* 1973). In the subsequent period it was widely accepted that the *ca.* 120 kDa monomer ('large' phytochrome) represented the undegraded, native chromoprotein, and once again much information on its molecular properties was accumulated (Pratt 1979, 1982).

Recently, however, we have shown that phytochrome from etiolated *Avena* is a homogeneous species with a monomeric molecular mass of 124 kDa, some 6–10 kDa larger than the heterogeneous mix of 118 and 114 kDa polypeptides that actually compose the '120' kDa preparations (Quail *et al.* 1981; Vierstra & Quail 1982*a*). Time-course and inhibitor studies have provided strong evidence that the 118 and 114 kDa species are derived from the 124 kDa molecule by post-homogenization proteolysis and that the P_r form is much more susceptible to this proteolysis than the P_{fr} form. Similar limited proteolysis has now also been documented in extracts of rye, corn, pea and zucchini seedlings, indicating the generality of the problem (Vierstra & Quail, unpublished; Kerscher & Nowitzki 1982). Because previous purification protocols have invariably stipulated rigorous maintenance of phytochrome in the P_r form throughout the procedure, it is highly likely that most of the data collected on the molecular properties of the purified photoreceptor have been obtained with partly degraded preparations (Pratt 1979, 1982).

Three lines of evidence indicate that 124 kDa phytochrome represents the native monomer in *Avena* and is not yet another proteolytic degradation product of a still larger polypeptide. First, the spectral properties, including the peak absorbance position (λ_{max}) of P_{fr}, are the same as those determined for phytochrome *in vivo* by difference spectroscopy (Vierstra & Quail 1982*b*). Second, like *Avena* phytochrome *in vivo*, the 124 kDa molecule exhibits no dark reversion (Vierstra & Quail 1983*a*). Third, the mobility of 124 kDa phytochrome is indistinguishable from that of the *in vitro* translation product of phytochrome mRNA, indicating the absence of both *in vivo* proteolytic processing and post-homogenization proteolysis (Bolton & Quail 1982). Thus the properties determined for the 124 kDa molecule would appear likely to reflect those of undegraded, undenatured phytochrome.

While at first sight the reduction in molecular mass occurring upon proteolytic conversion of the 124 kDa to the 118/114 kDa species might appear small and potentially unimportant, the evidence so far available indicates that a large number of phytochrome properties are altered significantly by this degradation (summarized in table 1). As well as changing the p*I* of the molecule (Vierstra & Quail 1982*a*), progressive proteolysis *in vitro* has been shown, by difference spectroscopy in crude extracts, to lead to a shift in the λ_{max} of P_{fr} from 730 to 722 nm (figure 1) (Vierstra & Quail 1982*b*). This observation explains the previously longstanding discrepancy between the P_{fr} λ_{max} measured *in vivo* and that of purified phytochrome (Everett & Briggs 1970). The data also provide a molecular interpretation for the empirical observations of Epel and coworkers, who found that phytochrome extracted as P_{fr} had a P_{fr} λ_{max} similar to that *in vivo* whereas extraction or incubation, or both, as P_r caused a shift in P_{fr} λ_{max} by about 10 nm to shorter wavelengths (Epel 1981; Baron & Epel 1982). These workers had interpreted their data to represent some form of 'activation' and 'deactivation' in the P_{fr} and P_r forms respectively.

Studies with purified 124 kDa phytochrome, obtained with a recently developed procedure

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(Vierstra & Quail 1983*a*), indicate additional spectral differences between the native and partly degraded species. The absorbance spectra of purified 124 kDa phytochrome (figure 2) differ from those of purified 118/114 kDa preparations in the P_{fr} λ_{max} positions both in the far red and in the blue regions and exhibit enhanced absorbance of P_{fr} at 730 nm relative both to the 673 nm shoulder of the red-generated spectrum and to the maximum absorbance of P_r at 666 nm (table 1). Purified 124 kDa phytochrome also exhibits negligible dark reversion with or without dithionite and has an enhanced quantum yield for the $P_r \rightarrow P_{fr}$ photoconversion relative to 118/114 kDa phytochrome and a photoequilibrium in red light of 86% P_{fr}, which is substantially higher than prevous reports for *Avena* (Vierstra & Quail 1983*a*, *b*; Pratt 1975). Differences in the circular dichroism (c.d.) spectra, indicative of differences in molecular structure between the 124 kDa and 118/114 kDa species, have also been detected (Vierstra, Hahn, Sarkar, Song & Quail, unpublished).

TABLE 1. PREVIOUS AND REVISED PHYTOCHROME PROPERTIES

	previous	revised	references
monomer molecular mass/kDa	118/114	124	1
p*I*	5.8/6.0	5.9	1
N-terminus	Lys, Ala	blocked	2,3
P_r λ_{max} (red)/nm	667	666	3, 4, 5, 6
P_{fr} λ_{max} (far red)/nm	722–724	730	3, 4, 5, 6
P_r λ_{max} (blue)/nm	382	379	3, 4
P_{fr} λ_{max} (blue)/nm	390	400	3, 4
$A^{P_{fr}}_{\lambda max}/A^{P_{fr}}_{shoulder}$	0.92–1.13	1.45	3, 4, 6
$A^{P_{fr}}_{\lambda max}/A^{P_r}_{\lambda max}$	0.43–0.48	0.58	3, 4, 6
spectral change ratio, $\Delta A_{red}/\Delta A_{far\,red}$	1.24–1.35	1.07	5, 7
P_r quantum yield (ϕ_r)	0.11	0.17	6, 8
P_{fr} quantum yield (ϕ_{fr})	0.12	0.10	6, 8
photoequilibrium in red light	0.75–0.79	0.86	6, 8
dark reversion *in vitro*	yes	minimal	3, 9
effect of ANS on photoconversion rate	altered	no effect	10, 11
Trp phosphorescence-quench by chromophore	$P_r > P_{fr}$	$P_r = P_{fr}$	11, 12
tetranitromethane bleaching $P_{fr} > P_r$	50-fold	8-fold	13, 14
c.d. spectra ΔA, 190–220 nm	$P_r = P_{fr}$	$P_r < P_{fr}$	11, 15
ΔA (rel. units), 700–750 nm	2	1	11, 16

References: 1, Vierstra & Quail (1982*a*); 2, Hunt & Pratt (1980*a*); 3, Vierstra & Quail (1983*a*); 4, Hunt & Pratt (1979*b*); 5, Vierstra & Quail (1982*b*); 6, Vierstra & Quail (1983*b*); 7, Pratt & Cundiff (1975); 8, Pratt (1975); 9, Pike & Briggs (1972); 10, Hahn & Song (1981); 11, Vierstra, Hahn, Sarkar, Song & Quail (unpublished); 12, Sarkar & Song (1982); 13, Hahn *et al.* (1980); 14, Hahn *et al.* (1983); 15, Tobin & Briggs (1973); 16, Song *et al.* (1979).

Experiments designed to probe the moleule for differences in the P_r and P_{fr} forms provide further evidence of molecular changes resulting from the limited proteolysis. Neither the effect of 8-anilinonaphthalene-1-sulphonate (ANS) on photoconversion rate (Hahn & Song 1981) nor the enhanced quenching of tryptophan phosphorescence by the chromophore in the P_r form (Sarkar & Song 1982) previously reported for the 118/114 kDa species is observed with 124 kDa phytochrome (Vierstra, Hahn, Sarkar, Song & Quail, unpublished). These data call into question the hypothesis of Song and coworkers (Hahn & Song 1981; Sarkar & Song 1982) that P_r–P_{fr} photoconversion results in movement of the chromophore relative to the protein with the exposure of a hydrophobic domain on the molecule. The rate of bleaching by tetranitromethane is 50-fold higher for P_{fr} than for P_r with 118/114 kDa phytochrome but only

8-fold higher with the 124 kDa molecule (Hahn *et al.* 1983). This result indicates that the chromophore is less reactive with the surrounding medium in the P_{fr} form of 124 kDa phytochrome than of 118/114 kDa phytochrome. The data do not permit a distinction between whether this difference represents a change in the intrinsic chemical reactivity of the chromophore or a change in spatial location or both.

The amino acid composition of 124 kDa phytochrome differs only minimally in molar proportions from that of 118/114 kDa preparations (Vierstra & Quail 1983*a*). The minor apparent differences that have been measured indicate a possible tendency toward slight depletion of Tyr, Phe, Val and Asx and slight enrichment of Thr, Arg and Met in the

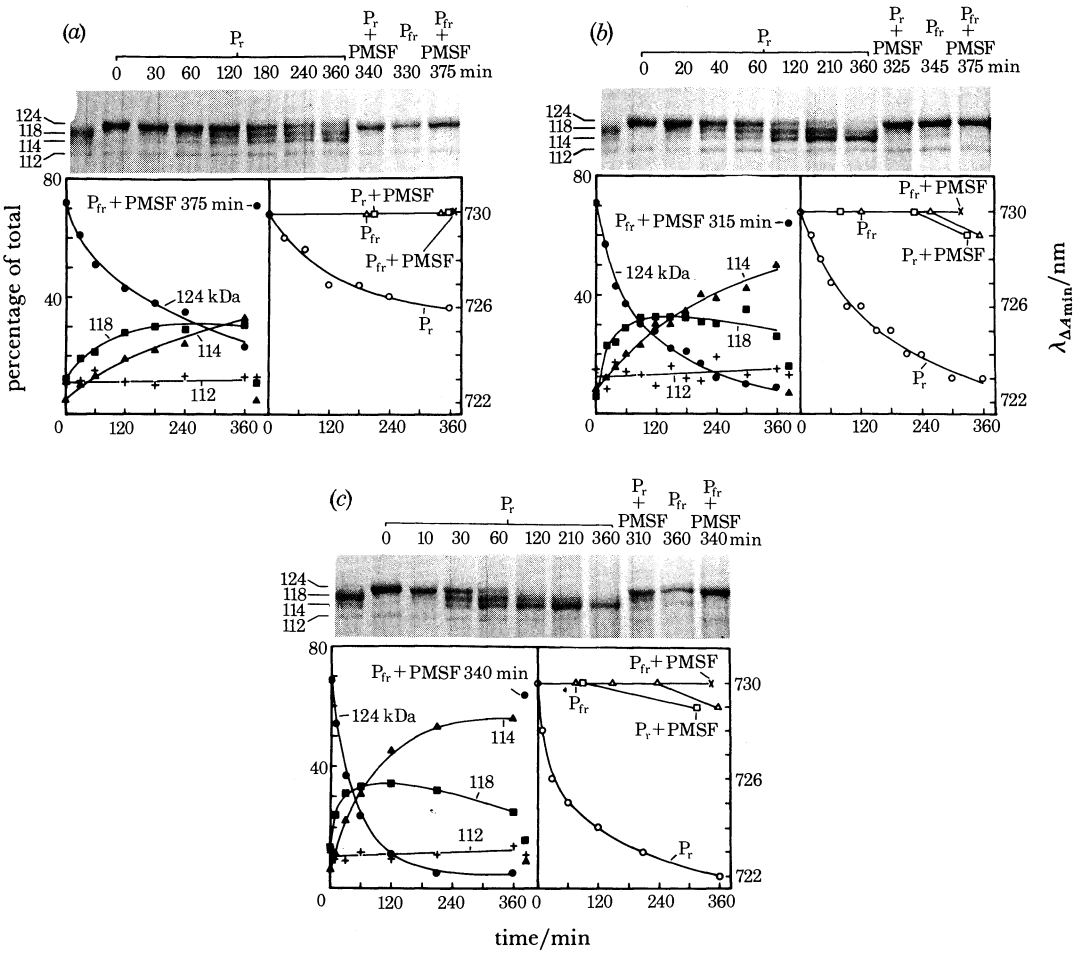


FIGURE 1. Time-course of the change in electrophoretic mobility and P_{fr} λ_{max} during incubation of *Avena* phytochrome as P_r in crude extracts from etiolated seedlings. Phytochrome was extracted in the P_{fr} form and then either retained as P_{fr} or reconverted to P_r in the 48000 *g* supernatant from the extract before incubation at 2 °C (*a*), 10 °C (*b*) and 20 °C (*c*) with or without the addition of 2 mM PMSF. At the times indicated difference spectra ($P_r - P_{fr}$) were recorded and phytochrome was immunoprecipitated in the P_{fr} form (in all cases) with antiphytochrome IgG-coated *S. aureus* cells (Ivarie & Jones 1979). The immunoprecipitates were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis in 5% (by mass) acrylamide gels (Laemmli 1970), and the percentage of phytochrome in each molecular mass species (124 (•), 118 (▪), 114 (▲) and 112 (+) kDa) was estimated from absorbance scans at 620 nm of the gels stained with Coomassie blue (left-hand panels). The P_{fr} λ_{max} determined from the difference spectrum (ΔA minimum) for each sample is also plotted as a function of time (right-hand panels). The first lane of each gel contains 1 μg of column-immonopurified phytochrome for comparison. (Data from Vierstra & Quail (1982*b*).)

proteolytically cleaved fragment relative to the average composition of the remainder of the molecule. The consequences of these differences to the properties of the 6–10 kDa polypeptide fragment are unknown. In contrast to 118/114 kDa preparations, which have been reported to contain a mixture of two N-terminal amino acids, Lys and Ala (Hunt & Pratt 1980*a*), 124 kDa phytochrome has a blocked N-terminus (Vierstra & Quail 1983*a*).

The changes in properties that occur upon this adventitous proteolytic conversion of 124 kDa to 118/114 kDa phytochrome have provided valuable information on the molecular structure of the photoreceptor. It is clear that the 6–10 kDa polypeptide fragment(s) is critical to the structural and spectral integrity of the molecule and thus potentially important to its functional

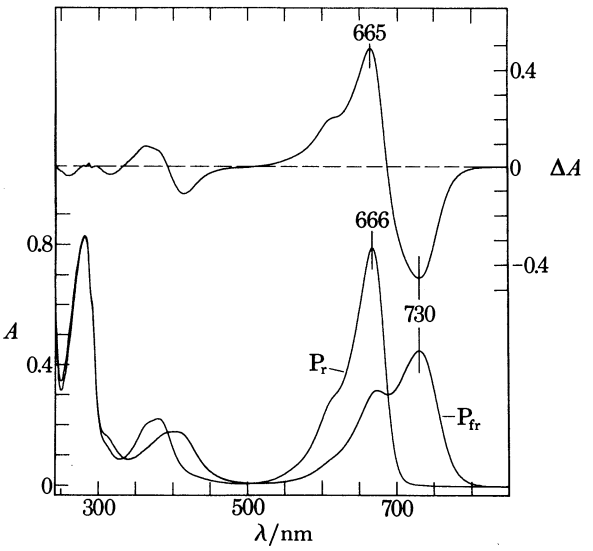


FIGURE 2. Absorbance and difference spectra of 124 kDa *Avena* phytochrome ($A_{666}/A_{280} = 0.97$). Absorbance spectra were measured after saturating red (P_{fr}) and far-red (P_r) irradiation, and the difference spectrum was obtained by subtracting the spectrum of P_{fr} from P_r. (Data from Vierstra & Quail (1983*a*).)

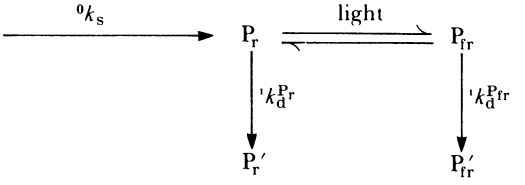
properties. The loss of the blocked N-terminus upon proteolysis indicates that at least part of this critical polypeptide domain is located at the N-terminus of the molecule. The differential susceptibility of the proteolytically sensitive site(s) in the P_r and P_{fr} forms indicates that phototransformation causes a change in molecular topology involving this domain. The report that the shift in P_{fr} λ_{max} with incubation as P_r in crude homogenates occurs in a wide range of species (Baron & Epel 1982) suggests a conservation of the site and its surrounding domain. The differential rates of bleaching of P_r and P_{fr} by tetranitromethane also reflect differences in chromophore chemistry and/or topology of the two forms.

AUTOREGULATION OF TRANSLATABLE mRNA LEVELS

A full understanding of the function of phytochrome in the living cell must include an understanding of the regulation of the levels of the photoreceptor. It has long been known that these levels are controlled both developmentally and by light. Phytochrome is synthesized *de novo* in the P_r form (Quail *et al.* 1973) accumulating in dark-grown tissue until a plateau is reached (Schäfer *et al.* 1972; Quail *et al.* 1973). This plateau represents a steady-state balance

between the synthesis and degradation of P_r (Quail *et al.* 1973). Transfer of tissue to the light results in a rapid decline in phytochrome levels because P_{fr} has a much greater rate of degradation (100-fold in *Cucurbita*) than P_r (Quail *et al.* 1973; Schäfer *et al.* 1975; Hunt & Pratt 1979*b*, 1980*b*; Pratt 1979). In plants transferred to continuous white light a new constant level is reached at 1–3% of the phytochrome initially present (Hunt & Pratt 1979*b*; Jabben & Deitzer 1978), presumably representing a new steady-state balance between P_r synthesis and P_{fr} degradation. Return of light-treated tissue to the dark leads to a reaccumulation of phytochrome in the P_r form by continued synthesis *de novo* (Hunt & Pratt 1980*b*; Quail *et al.* 1973; Schäfer *et al.* 1975).

These and other data have led to the generalized view of the phytochrome system *in vivo* shown in scheme 1, where ${}^0k_s$ is a zero-order rate constant of synthesis; ${}^1k_d^{P_r}$ and ${}^1k_d^{P_{fr}}$ are first-order rate constants of degradation of P_r and P_{fr} respectively, with ${}^1k_d^{P_r} \ll {}^1k_d^{P_{fr}}$; and P_r' and P_{fr}' are degradation products of P_r and P_{fr}. In this view phytochrome levels are considered to be modulated strictly at the protein level by the disparate rate constants of degradation for P_r and P_{fr} against a constant background rate of synthesis determined by an unchanging ${}^0k_s$. Clearly implied in this scheme is a constant level of translatable phytochrome mRNA supporting a constant rate of *de novo* synthesis of the chromoprotein.



Scheme 1

Recent studies on the cell-free synthesis of the phytochrome apoprotein have led us to consider a second level of control. Whereas translatable phytochrome mRNA is readily detectable in etiolated tissue, none is detectable in green tissue grown in continuous white light (Quail *et al.* 1983; Hershey *et al.* 1982; Gottmann & Schäfer 1982). When the levels of translatable phytochrome mRNA are monitored after irradiation of etiolated tissue with 5 s of red light, a very rapid decline in this translatable mRNA is observed (figure 3) (Colbert *et al.* 1983). The decline is detectable within 15–30 min, a 50% reduction occurs within 50–60 min and more than 95% reduction occurs within 2 h (figure 3*b*). The effect of the red light pulse is reversed by an immediately subsequent far-red pulse to the level of the far-red light control, indicating that phytochrome exerts autoregulatory control over its translatable mRNA level (table 2). Red light dose–response curves show that the response is senstive to very low levels of P_{fr} (Colbert *et al.* 1983). Conversion of less than 1% of the total cellular phytochrome to P_{fr} induces about 60% of the maximum response, and 20% P_{fr} saturates the response.

These data indicate that scheme 1 should be revised as shown in scheme 2 to reflect the dual control system involved. The photoconversion of phytochrome to P_{fr} not only enhances the degradation of the chromoprotein but also reduces its rate of synthesis by causing a decrease in the level of translatable phytochrome mRNA. This negative feedback modulation of translatable mRNA levels appears to be reversible upon depletion of P_{fr} in extended darkness. Gottmann & Schäfer (1982) have reported the reappearance of detectable levels of translatable phytochrome mRNA in green *Avena* returned to the dark for 10–24 h.

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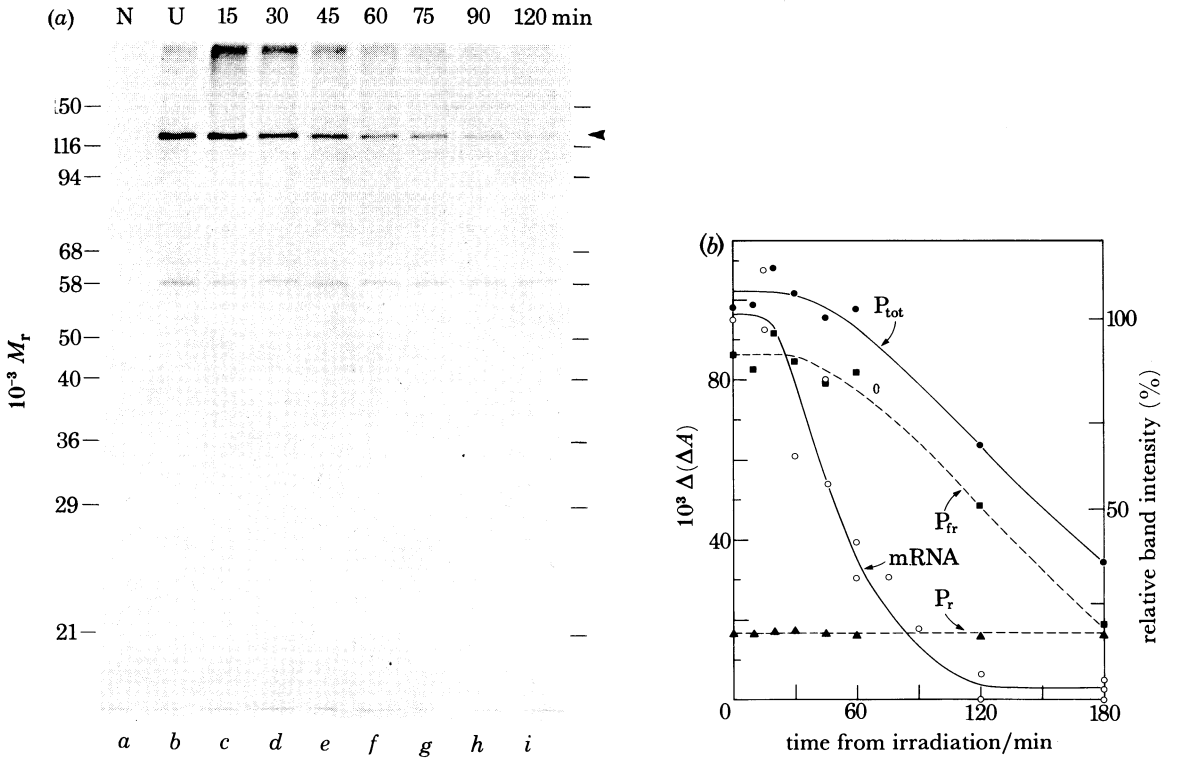


FIGURE 3. The effect of red irradiation on the levels of phytochrome and phytochrome mRNA in etiolated *Avena* shoots. (*a*) Time course of the decrease in translatable phytochrome mRNA in darkness after a 5 s saturating red irradiation. At the times indicated poly(A) RNA was isolated (Colbert *et al.* 1983) and translated (Pelham & Jackson 1976), and the phytochrome apoprotein (arrow) was immunoprecipitated (Ivarie & Jones 1979). The immunoprecipitates were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemmli 1970) and fluorographed (Laskey 1980). Lane *A*, non-immune control (*N*), unirradiated tissue. Lanes *b*–*i*, antiphytochrome IgG immunoprecipitates. Lane *b*, unirradiated tissue (*U*). Lanes *c*–*i*, 5 s of red light followed by incubation in the dark for: lane *c*, 15 min; lane *d*, 30 min; lane *e*, 45 min; lane *f*, 60 min; lane *g*, 75 min; lane *h*, 90 min; lane *i*, 120 min. The minor band at *ca.* 58 kDa is thought to be a contaminant recognized by non-phytochrome antibodies in the antiphytochrome IgG preparations, because this band is not observed in immunoprecipitates from fractions containing phytochrome mRNA after size fractionation of poly(A) RNA on sucrose gradients (see figure 5). (*b*) Time-course of the change in cellular phytochrome and translatable phytochrome mRNA in etiolated shoots in darkness after a saturating red (5 s) irradiation. At the times indicated either poly(A) RNA was isolated and translated, or phytochrome was extracted and measured. The *in vitro* synthesized phytochrome apoprotein was immunoprecipitated, subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis, and quantitated by scanning fluorographs of these gels (○). Data points are from four different experiments including that in (*a*) and are expressed as a percentage of the initial levels in the unirradiated control in each case. Phytochrome ($10^3\Delta(\Delta A)$) was measured spectrophotometrically in crude extracts with $CaCO_3$ as a scattering agent. P_{tot}, total amount of spectrally detectable phytochrome (●); P_r (▲), P_{fr}(■), amounts of the two spectral forms that comprise P_{tot}. The levels of P_r and P_{fr} at time zero are those immediately after the 5 s red irradiation. Data points are the means of two different experiments in which each time point was duplicated. (Data from Colbert *et al.* (1983).)

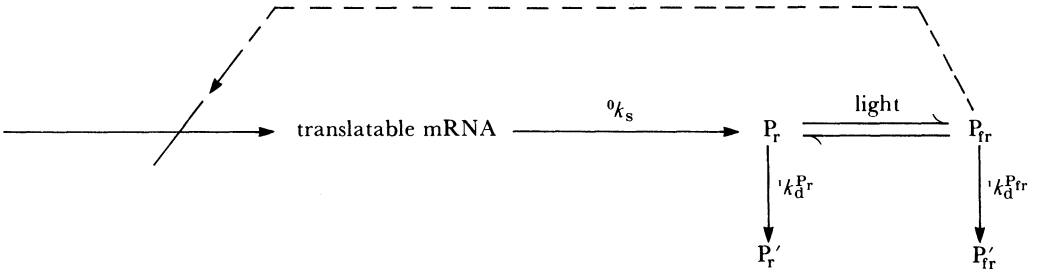
There are several implications of these findings. First, whereas short-term fluctuations in phytochrome levels in response to light involve control predominantly at the protein level by the differential turnover rates of P_r and P_{fr}, in the long term the decreased rate of P_r synthesis needs to be accounted for. In particular, attempts to perform quantitative physiological studies under continuous irradiation or under diurnal light–dark cycles will need to take into consideration the expected fluctuating rates of synthesis as well. Second, the level of phytochrome measured in green tissue in continuous light (1–3% of the etiolated tissue level (Hunt & Pratt

TABLE 2. AUTOREGULATORY CONTROL OF TRANSLATABLE PHYTOCHROME mRNA LEVELS

(Etiolated *Avena* seedlings were irradiated as indicated and returned to the dark for 3 h before poly(A) RNA was isolated and translated. Immunoprecipitates were prepared by using antiphytochrome IgG, subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis and fluorographed. The relative intensities of the phytochrome apoprotein band in each treatment were determined by scanning the fluorographs as in figure 3. Data from Colbert *et al.* (1983).)

treatment	translatable phytochrome mRNA level (relative units)
unirradiated control	100
5 s red + 3 h dark	5
5 s red + 6 s far red + 3 h dark	34
6 s far red + 3 h dark	37

1979*b*; Jabben & Deitzer 1978)) (*a*) appears to be higher than expected of the combined effect of the more than 20-fold decrease in rate of synthesis (figure 3*b*) and 100-fold increase in rate of degradation (Quail *et al.* 1973) that should apparently accompany P_{fr} formation, and (*b*) appears to be higher than expected given the fact that no phytochrome apoprotein is detectable



SCHEME 2

among the cell-free translation products of green-tissue poly(A) mRNA (Quail *et al.* 1983; Gottmann & Schäfer 1982). One potential explanaton of this apparent discrepancy that is being explored is that the phytochrome detected in green tissue is a second gene product, immunologically distinct from that predominating in etiolated tissue. Third, phytochrome mRNA (figure 3), together with protochlorophyllide reductase mRNA (Apel 1981), exhibits the most rapid change in phytochrome-regulated mRNA (15 min lag) so far reported. The rapidity of this autoregulation thus ironically makes phytochrome itself an attractive system with which to investigate the more general question of the mechanisms underlying phytochrome-regulated gene expression.

cDNA SYNTHESIS AND CLONING

We are in the process of cloning phytochrome cDNA for several purposes including the deduction of the primary sequence of the phytochrome polypeptide, the monitoring of potential developmental and light-induced changes in the physical abundance of phytochrome mRNA and the isolation and characterization of the phytochrome gene(s). This task has proved not to be simple because of the unexpectedly low abundance of the phytochrome mRNA. In contrast to the chromoprotein, which composes about 0.5% of the soluble protein in etiolated *Avena* shoots (Bolton 1979; Vierstra & Quail 1983*a*), translatable phytochrome mRNA is less

than 0.005% of the poly(A) RNA in this tissue (Colbert *et al.* 1983; Hershey *et al.* 1982). By analogy with estimates for soybean tissue (Fischer & Goldberg 1982) this value corresponds to only about 25 phytochrome mRNA molecules per cell. Essentially all cDNA clones for identified plant nuclear gene products thus far isolated correspond to mRNAs that are in the medium to high abundance category (2–50% of the cellular mRNA mass) (see, for example, Bedbrook *et al.* 1980; Bernal-Lugo *et al.* 1981; Broglie *et al.* 1981; Chandler *et al.* 1983; Geraghty *et al.* 1981; Meinke *et al.* 1981). Moreover, although there is an increasing number of examples from mammalian systems where cDNAs for low abundance mRNAs have been isolated (Edge & Markum 1982; Korman *et al.* 1982; Kraus & Rosenberg 1982; Parnes *et al.* 1981; Sood *et al.* 1981), these are still relatively few and represent mRNAs that are 0.01–0.2% of the total cellular mRNA, above that of phytochrome mRNA.

Our initial attempts to overcome this handicap have involved exploiting the relatively large size of the phytochrome mRNA to provide enrichment by size fractionation and using a synthetic oligodeoxynucleotide complementary to part of the mRNA sequence as a hybridization probe for screening a cDNA library for phytochrome clones. Poly(A) RNA from etiolated *Avena* tissue has been fractionated by rate-zonal sucrose gradient centrifugation, and the fractions containing phytochrome mRNA have been located by *in vitro* translation and immunoprecipitation (figure 4). This procedure provides an enrichment of 25–40-fold in a single pass and a further 4–5-fold by a second gradient. Although phytochrome mRNA still composes only 1.0–1.5% of the mRNAs in the most enriched fraction, it has been used to synthesize cDNA (figure 5), the double-stranded cDNA has been inserted into the *Hin*d III site of pBR322 by using synthetic linkers, and the recombinant plasmids have been used to transform *Escherichia coli* RR1.

The successful use of synthetic oligonucleotide probes to isolate cDNA clones representing the low-abundance interferon mRNA (Edge & Markum 1982) encouraged us to adopt this strategy as the most direct and efficient means of identifying phytochrome cDNA clones. The sequence of the probe used was determined from the only phytochrome protein sequence available, a segment of 11 amino acids associated with the chromophore (figure 6). Because of the limited extent of the known polypeptide sequence and the generally high degree of degeneracy in the corresponding nucleotide sequence, it was necessary to synthesize a wobble mix of 24 different 14-mers to accommodate all possible permutations.

Initial attempts to use the ^{32}P-labelled 14-mers directly as colony hybridization probes failed to identify colonies hybridizing strongly above background, possibly because the average G–C content of the wobble mixture is only 33%. The differential between the stringency conditions permitting specific and non-specific duplex formation is expected to be very narrow for this oligonucleotide. It was thus decided to generate longer probes of much higher specific activity by using the 14-mers as selective primers for cDNA synthesis directed by reverse transcriptase with poly(A) RNA enriched for phytochrome mRNA as a template. Some 24 positively hybridizing colonies were isolated from the cDNA library in this fashion and subjected to a Northern blot secondary screening procedure. Adjacent tracks of poly(A) RNA – one from unirradiated tissue and one from tissue exposed to light to deplete the phytochrome mRNA – were probed with nick-translated plasmids from the various positive clones. Plasmids carrying phytochrome inserts would be expected to hybridize to an mRNA that is depleted in the poly(A) fraction from light-exposed tissue (assuming transcriptional control) and that migrates as a 3.5–4 kilobase species. No clone with both the required characteristics was located.

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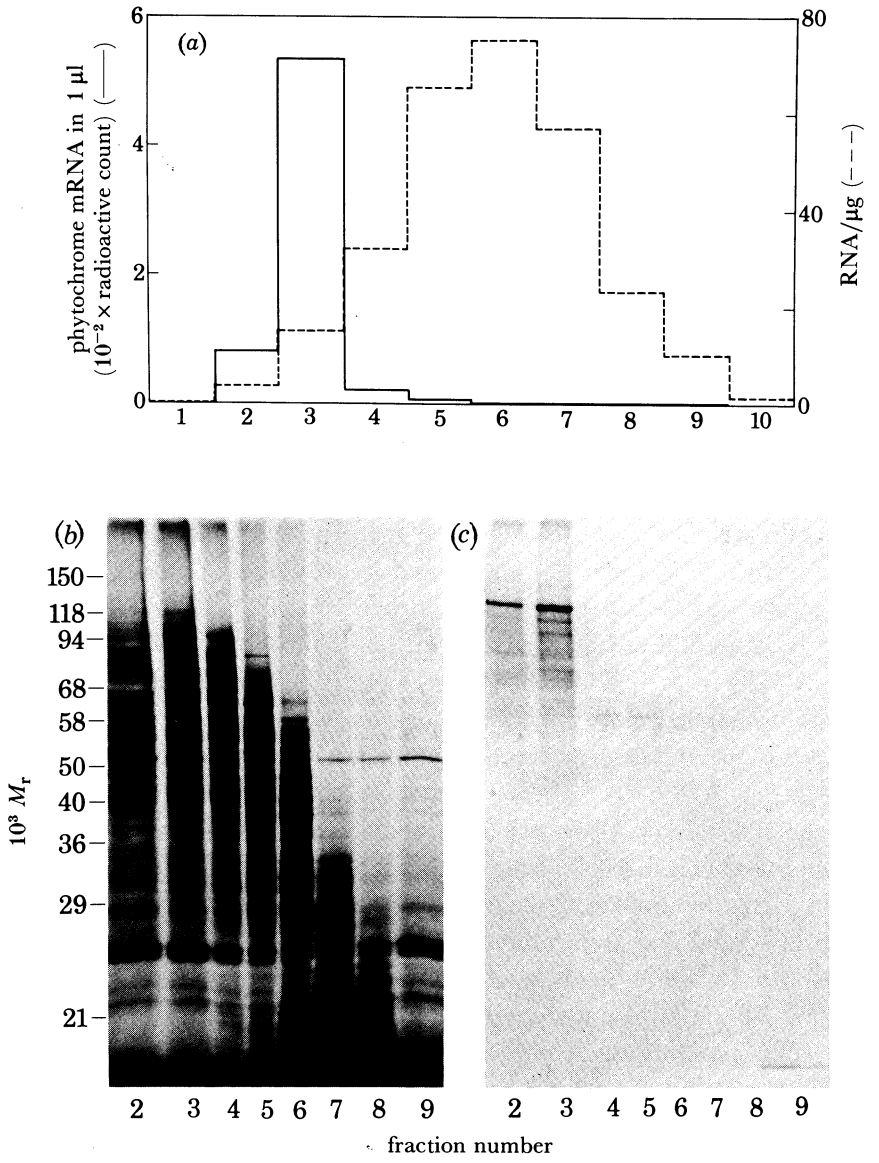

FIGURE 4. Fractionation of poly(A) RNA from etiolated *Avena* tissue. Poly(A) RNA was enriched for phytochrome mRNA by centrifugation through a linear sucrose gradient (50–300 g l^{-1}). After fractionation of the gradient, mRNA from each fraction was recovered and translated in an mRNA-dependent reticulocyte lysate. (*a*) – – –, Distribution of RNA in the gradient as determined by A_{260}; ——, distribution of phytochrome mRNA in the gradient as determined by translation and immunoprecipitation with antiphytochrome IgG. (*b*) Sodium dodecyl sulphate polyacrylamide gel electrophoresis and fluorography of translation products synthesized in response to gradient fractionated mRNA by a nuclease-inactivated reticulocyte lysate. Equal amounts of radioactivity were loaded on each lane. (*c*) Sodium dodecyl sulphate polyacrylamide gel electrophoresis of products immunoprecipitated by antiphytochrome IgG from the total translation products of the gradient fractions shown in (*b*). Fractions enriched for translatable phytochrome mRNA have been recentrifuged on a linear sucrose gradient (150–300 g l^{-1}) to further enrich the phytochrome mRNA. The phytochrome mRNA in the peak fraction of this second gradient composed about 1 % of the poly(A) in this fraction, thus representing about a 200-fold enrichment relative to the initial poly(A) fraction. (Data from Quail *et al.* (1983).)

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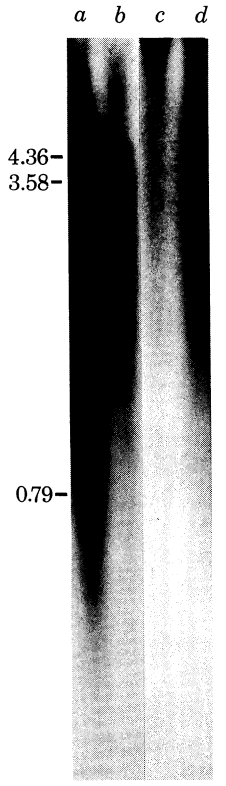

FIGURE 5. cDNA synthesized from the fraction most enriched in phytochrome mRNA from the second pass sucrose gradient referred to in figure 4. High molecular mass double-stranded (ds) cDNA was synthesized either by a modification of the method of Wickens *et al.* (1978) or by a modification of the method of Land *et al.* (1981). The size of cDNA made by each procedure was measured on 1.5% (by mass) agarose gels. In both cases the cDNA averaged over 2.8 kilobase pairs (lane *a*, Land *et al.*; lane *b*, Wickens *et al.*), with both containing cDNA corresponding to the upper molecular mass limit of the RNA used to direct first strand synthesis. ds cDNA was more than 95% S_1-resistant (lanes *c* and *d*). ^{32}P-labelled *Hin*d III linkers were blunt-end ligated to the cDNA shown in lane *b*. The cDNA was digested with *Hin*d III, separated from the linkers, and ligated into phosphatase-treated *Hin*d III digested pBR 322. The numbers at the left are kilobase pairs.

A possible reason for these results is that the temperature and salt conditions required for the reverse transcriptase reaction cause non-specific priming of cDNA synthesis. Oligonucleotides specific for only one mRNA have been shown in other systems to prime cDNA synthesis from a number of different mRNA species (Edge & Markum 1982). Our positive clones were found to be complementary to mRNAs of various sizes from *ca.* 1 to 7.5 kilobases, indicating a high level of this non-specific priming, probably exacerbated by the A–T-rich nature of the oligonucleotide.

The difficulties experienced with the synthetic probe and the observation made during this work that translatable phytochrome mRNA rapidly declines to less than one-twentieth of its original level upon P_{fr} formation has led us recently to explore a more conventional 'dark versus light' difference screening procedure. This approach has been used successfully to isolate cDNA clones for ribulose bisphosphate carboxylase small subunit (Bedbrook *et al.* 1980), chlorophyll *a*/*b* protein (Apel 1982) and protochlorophyllide reductase (Apel 1982). The strategy is to screen replica arrays of colonies with probes constructed separately from mRNA isolated from dark- and from light-treated tissue. Colonies displaying different degrees of hybridization with

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(*a*)

							chromophore					
protein N –		Leu	Arg	Ala	Pro	His	Ser	Cys	His	Leu	Gln	Tyr – C
mRNA	5′ –	CUU	CGU	GCU	CCU	CAU	UCU	UGU	CAU	CUU	CAA	UAU – 3′
		CUC	CGC	GCC	CCC	CAC	UCC	UGC	CAC	CUC	CAG	UAC
		CUA	CGA	GCA	CCA		UCA			CUA		
		CUG	CGG	GCG	CCG		UCG			CUG		
			AGA				AGU			UUA		
			AGG				AGC			UUG		

(*b*)

protein N –		Cys	His	Leu	Gln	Tyr	– C
mRNA	5′ –	UGU	CAU	CUU	CAA	UAU	– 3′
		UGC	CAC	CUC	CAG	UAG	
				CUA			
				CUG			
				UUA			
				UUG			

oligonucleotide 14-mer (+) strand

3′ – AC(A/G) GT(A/G) [GA(A/T/C/G) … AA(T/C)] GTT AT – 5′

↑ *

FIGURE 6. Known amino acid sequence adjacent to the phytochrome chromophore (Fry & Mumford 1971; Lagarias & Rapoport 1980) and the corresponding mRNA sequence used to design a synthetic oligonucleotide probe. (*a*) Entire known sequence. (*b*) Carboxy-terminus sequence used to specify synthetic oligonucleotide sequence. This region exhibits the least degeneracy available in the sequence. The 24 different 14-mer sequences synthesized in the wobble mix are indicated at the bottom. The arrow indicates the single potential mismatch in the sequence where only T was used instead of the prescribed T/C ambiguity.

the two probes are tentatively identified as light-regulated. Although this approach relies on the as-yet untested assumption that the mass rather than the translatability of phytochrome mRNA is controlled, precedent with other light-regulated gene products indicates that this is a reasonable first assumption (Apel 1982; Bedbrook *et al.* 1980; Everett *et al.* 1982). Figures 7 and 8 show the relative levels of translatable phytochrome mRNA in gradient fractionated poly(A) RNA from unirradiated and from irradiated tissues. We have constructed twin cDNA probes from the gradient fractions enriched (figure 7) and depleted (figure 8) in phytochrome mRNA and are currently screening the cDNA library.

CONCLUSIONS

Our concepts of the phytochrome molecule and the regulation of its levels *in vivo* are currently undergoing rapid revision. Investigation of the properties of the newly purified 124 kDa phytochrome will, it is hoped, yield an accurate picture of the functional properties of the native photoreceptor. Moreover, whereas at first it may be tempting to dismiss data obtained with the 118/114 kDa phytochrome as obsolete, experience so far indicates that judicious comparisons with the 124 kDa molecule can be expected to provide further valuable insights into the molecular structure of the chromoprotein. The discovery that phytochrome exerts negative feedback control over its own mRNA levels is interesting teleologically and has unexpectedly

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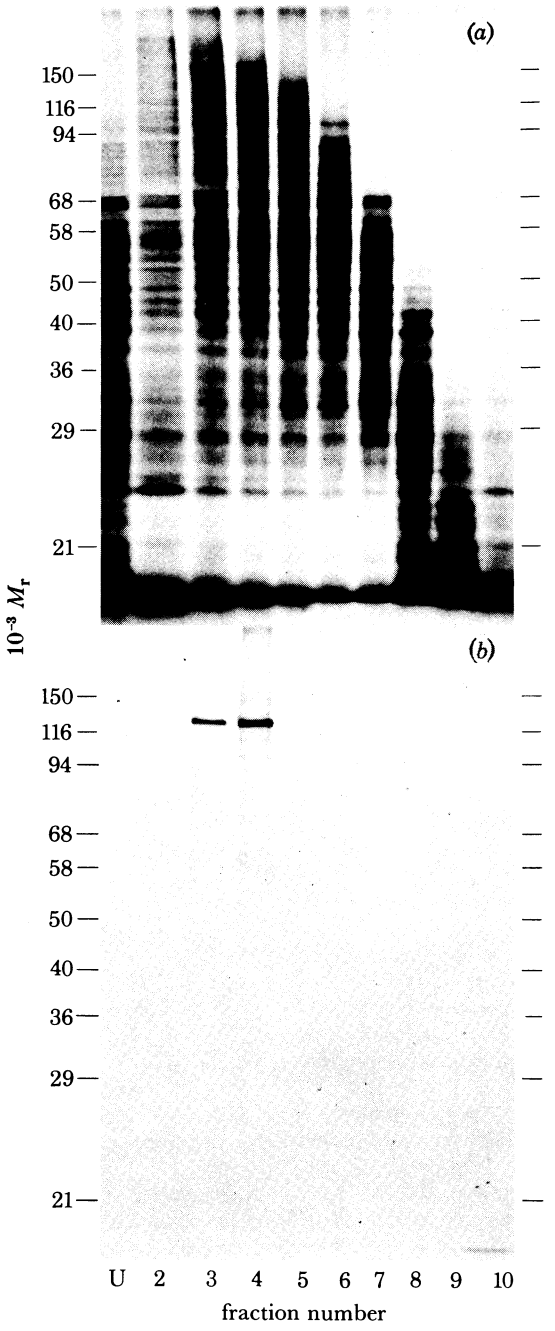

FIGURE 7. Sucrose gradient (50–300 g l^{-1}) fractionation of polysomal poly(A) RNA from etiolated, unirradiated *Avena* shoots. (*a*) Total translation products from each fraction. (*b*) Immunoprecipitates prepared from each fraction with antiphytochrome IgG. Phytochrome mRNA, concentrated in fractions 3 and 4, is enriched 10–20-fold relative to initial unfractionated poly(A) RNA.

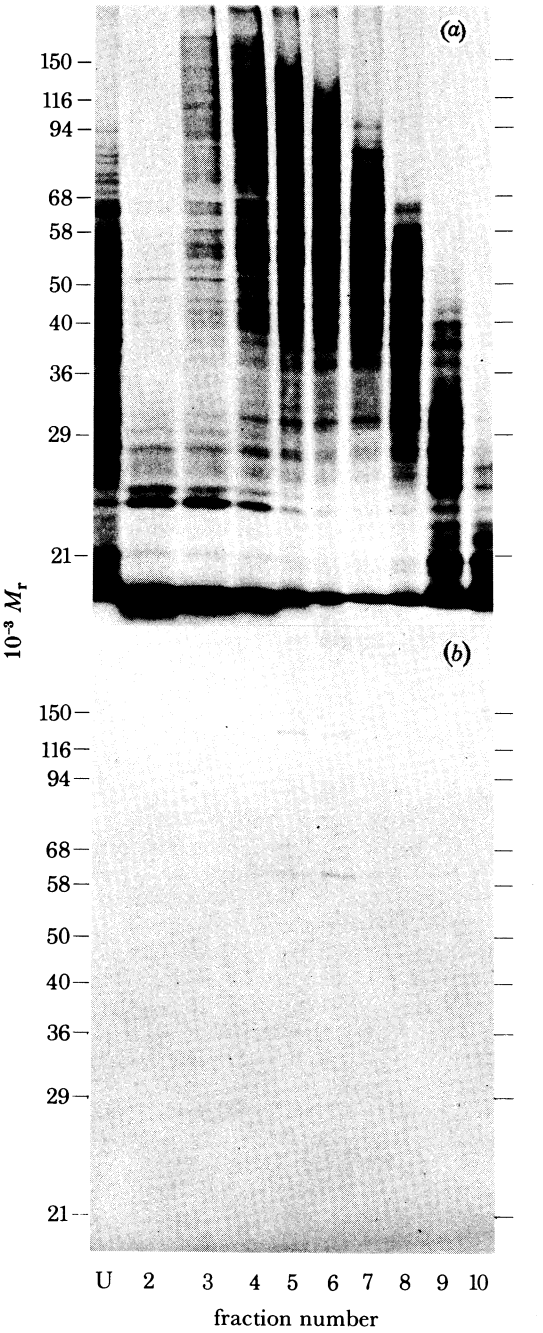

FIGURE 8. Sucrose gradient (50–300 g l^{-1}) fractionation of polysomal poly(A) RNA from etiolated oats irradiated with 5 min of red light and returned to the dark for a further 3 h before harvest. (*a*) Total translation products from each fraction. (*b*) Immunoprecipitates prepared from each fraction with antiphytochrome IgG. Phytochrome mRNA is in fractions 5 and 6 at a considerably lower level than in comparable fractions from unirradiated tissue (figure 7).

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provided an attractive system for investigating phytochrome-regulated gene expression. It seems reasonable that plants in the light may no longer need to synthesize the photoreceptor at the rates prevailing in seedlings before their first exposure to light. The relatively high levels of the photoreceptor observed in dark-grown plants may serve a light-scavenging function in the pre-emergent seedling, a function no longer needed after de-etiolation. The apparent retention in light-grown plants of the capacity to reinstate higher rates of synthesis upon P_{fr} depletion in prolonged darkness or under shaded conditions may serve a role in the photoperiod-monitoring and shade-detecting systems of plants. Experimentally, the rapid autoregulation of translatable phytochrome mRNA permits attention to be focused on a relatively short period in attempts to define the sequence of events between signal perception and altered gene expression.

We thank L. H. Pratt for initial assistance in producing antiphytochrome IgG, W. R. Briggs in whose laboratory the antiphytochrome IgG was produced and M. G. Murray for valuable discussions. The work was supported in part by National Science Foundation grants PCM 8003921, PCM 7723584, PCM 7514161, Department of Energy grant no. DE-AC02-81ER10903 and U.S. Department of Agriculture, Science and Education Administration grant no. 59-2551-1-1-744-0. J.T.C. was supported as a predoctoral trainee by National Institutes of Health grant no. 5T32GM07215.

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Discussion

W. Rüdiger (*Botanisches Institut der Universität München, F.R.G.*). The absorption maximum of P_{fr} has been reported to be at about 730 nm for native phytochrome but at about 720 nm for all forms of proteolytically degraded phytochrome. We found, however, that the absorption maximum of degraded phytochrome shifts from 720 nm (room temperature) to 730 nm at low temperature, e.g. at −40 °C. The influence of the small part of the peptide chain that is split off by the first proteolysis can therefore be described in the first approximation as 'freezing' of the chromophore.

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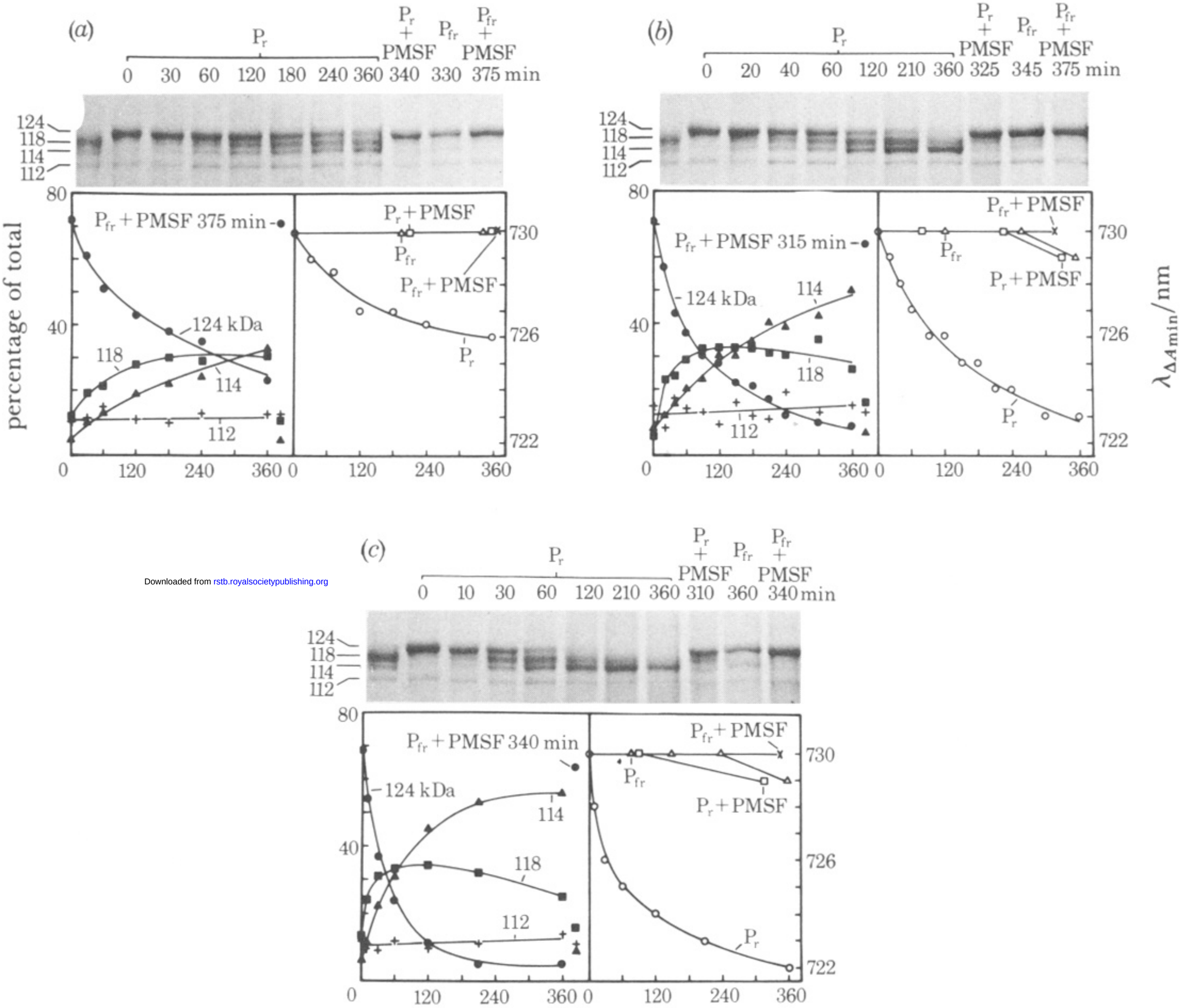


GURE 1. Time-course of the change in electrophoretic mobility and P_{fr} λ_{max} during incubation of *Avena* phytochrome as P_r in crude extracts from etiolated seedlings. Phytochrome was extracted in the P_{fr} form and then either retained as P_{fr} or reconverted to P_r in the 48000 *g* supernatant from the extract before incubation at 2 °C (*a*), 10 °C (*b*) and 20 °C (*c*) with or without the addition of 2 mM PMSF. At the times indicated difference spectra ($P_r - P_{fr}$) were recorded and phytochrome was immunoprecipitated in the P_{fr} form (in all cases) with antiphytochrome IgG-coated *S. aureus* cells (Ivarie & Jones 1979). The immunoprecipitates were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis in 5% (by mass) acrylamide gels (Laemmli 1970), and the percentage of phytochrome in each molecular mass species (124 (•), 118 (■), 114 (▲) and 112 (+) kDa) was estimated from absorbance scans at 620 nm of the gels stained with Coomassie blue (left-hand panels). The P_{fr} λ_{max} determined from the difference spectrum (ΔA minimum) for each sample is also plotted as a function of time (right-hand panels). The first lane of each gel contains 1 μg of column-immonopurified phytochrome for comparison. (Data from Vierstra & Quail (1982*b*).)

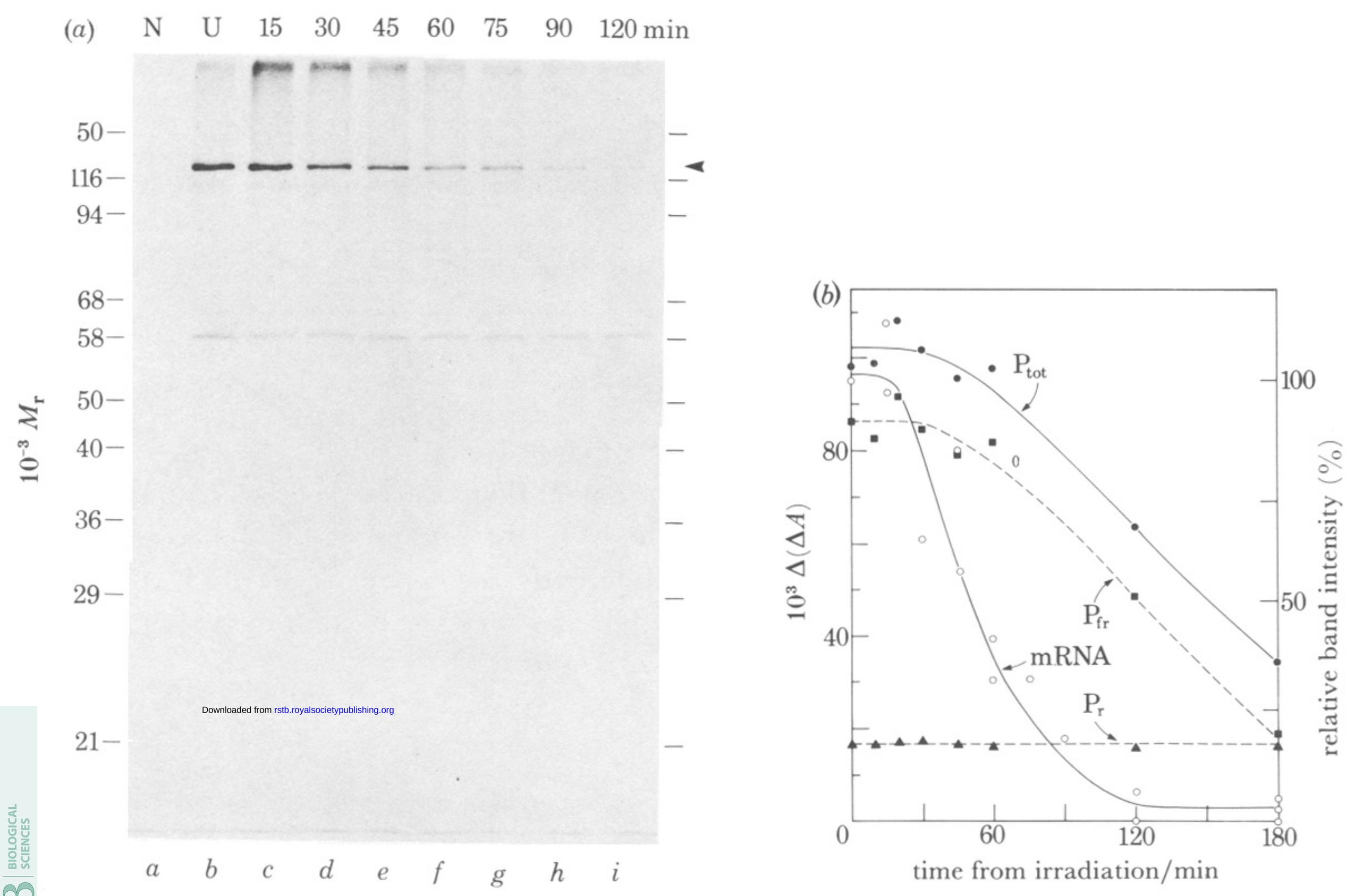
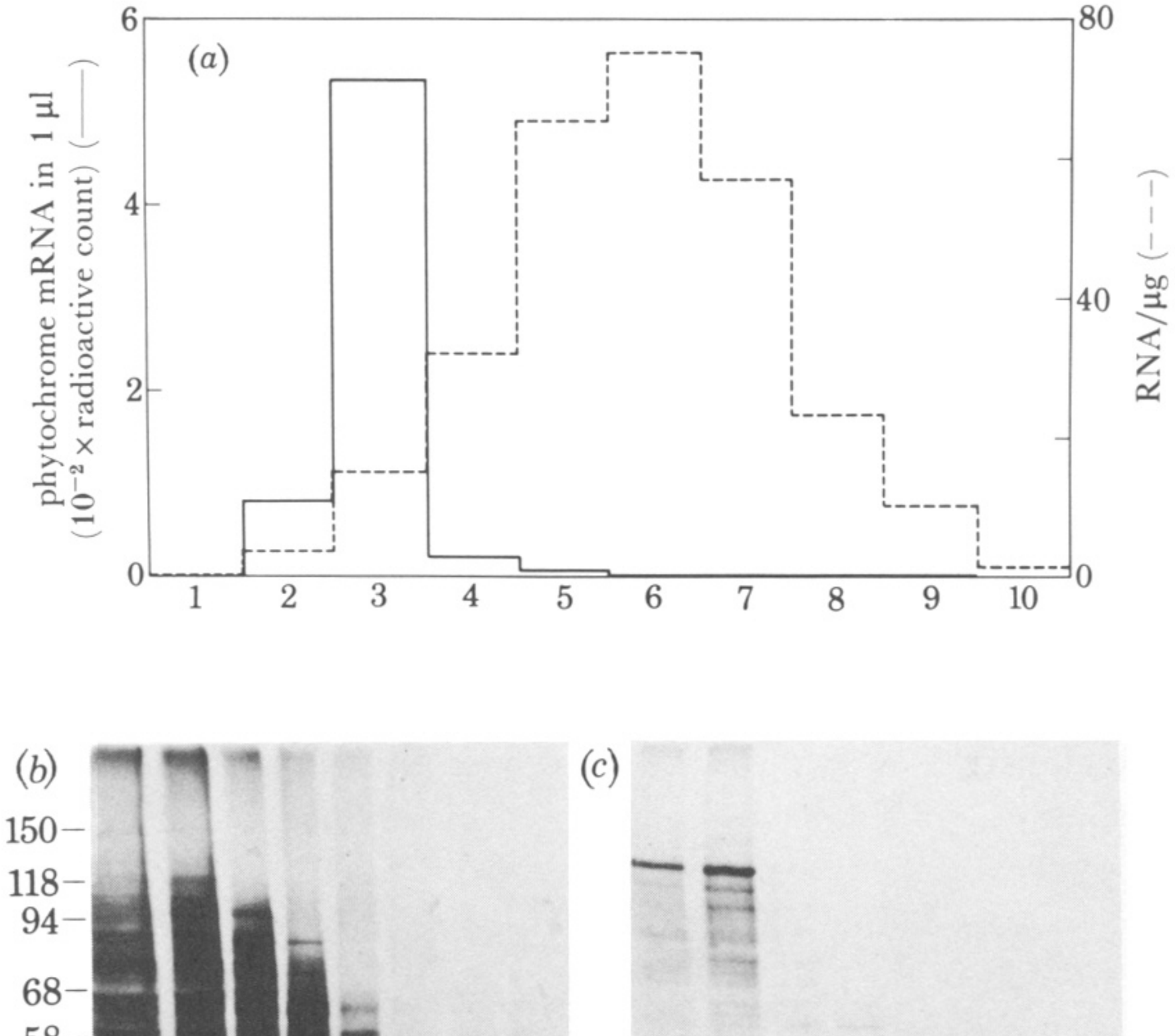


FIGURE 3. The effect of red irradiation on the levels of phytochrome and phytochrome mRNA in etiolated *Avena* shoots. (*a*) Time course of the decrease in translatable phytochrome mRNA in darkness after a 5 s saturating red irradiation. At the times indicated poly(A) RNA was isolated (Colbert *et al.* 1983) and translated (Pelham & Jackson 1976), and the phytochrome apoprotein (arrow) was immunoprecipitated (Ivarie & Jones 1979). The immunoprecipitates were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemmli 1970) and fluorographed (Laskey 1980). Lane *A*, non-immune control (*N*), unirradiated tissue. Lanes *b*–*i*, antiphytochrome IgG immunoprecipitates. Lane *b*, unirradiated tissue (*U*). Lanes *c*–*i*, 5 s of red light followed by incubation in the dark for: lane *c*, 15 min; lane *d*, 30 min; lane *e*, 45 min; lane *f*, 60 min; lane *g*, 75 min; lane *h*, 90 min; lane *i*, 120 min. The minor band at *ca.* 58 kDa is thought to be a contaminant recognized by non-phytochrome antibodies in the antiphytochrome IgG preparations, because this band is not observed in immunoprecipitates from fractions containing phytochrome mRNA after size fractionation of poly(A) RNA on sucrose gradients (see figure 5). (*b*) Time-course of the change in cellular phytochrome and translatable phytochrome mRNA in etiolated shoots in darkness after a saturating red (5 s) irradiation. At the times indicated either poly(A) RNA was isolated and translated, or phytochrome was extracted and measured. The *in vitro* synthesized phytochrome apoprotein was immunoprecipitated, subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis, and quantitated by scanning fluorographs of these gels (◦). Data points are from four different experiments including that in (*a*) and are expressed as a percentage of the initial levels in the unirradiated control in each case. Phytochrome ($10^3\Delta(\Delta A)$) was measured spectrophotometrically in crude extracts with $CaCO_3$ as a scattering agent. P_{tot}, total amount of spectrally detectable phytochrome (•); P_r (▲), P_{fr}(▪), amounts of the two spectral forms that comprise P_{tot}. The levels of P_r and P_{fr} at time zero are those immediately after the 5 s red irradiation. Data points are the means of two different experiments in which each time point was duplicated. (Data from Colbert *et al.* (1983).)



IGURE 4. Fractionation of poly(A) RNA from etiolated *Avena* tissue. Poly(A) RNA was enriched for phytochrome mRNA by centrifugation through a linear sucrose gradient (50–300 g l^{-1}). After fractionation of the gradient, mRNA from each fraction was recovered and translated in an mRNA-dependent reticulocyte lysate. (*a*) – – –, Distribution of RNA in the gradient as determined by A_{260}; —, distribution of phytochrome mRNA in the gradient as determined by translation and immunoprecipitation with antiphytochrome IgG. (*b*) Sodium dodecyl sulphate polyacrylamide gel electrophoresis and fluorography of translation products synthesized in response to gradient fractionated mRNA by a nuclease-inactivated reticulocyte lysate. Equal amounts of radioactivity were loaded on each lane. (*c*) Sodium dodecyl sulphate polyacrylamide gel electrophoresis of products immunoprecipitated by antiphytochrome IgG from the total translation products of the gradient fractions shown in (*b*). Fractions enriched for translatable phytochrome mRNA have been recentrifuged on a linear sucrose gradient (150–300 g l^{-1}) to further enrich the phytochrome mRNA. The phytochrome mRNA in the peak fraction of this second gradient composed about 1 % of the poly(A) in this fraction, thus representing about a 200-fold enrichment relative to the initial poly(A) fraction. (Data from Quail *et al.* (1983).)

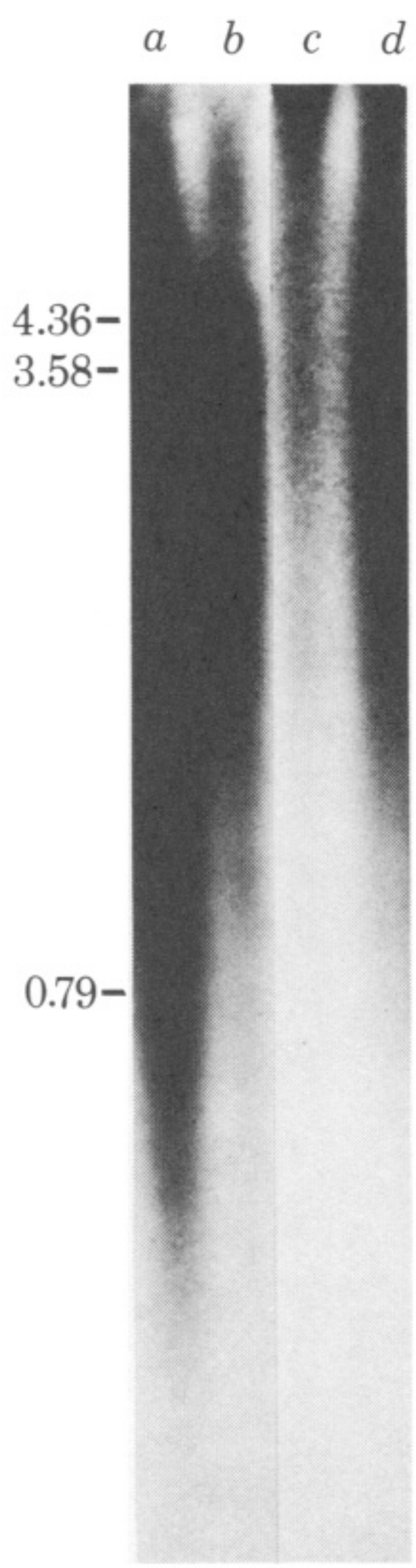


FIGURE 5. cDNA synthesized from the fraction most enriched in phytochrome mRNA from the second pass sucrose gradient referred to in figure 4. High molecular mass double-stranded (ds) cDNA was synthesized either by a modification of the method of Wickens *et al.* (1978) or by a modification of the method of Land *et al.* (1981). The size of cDNA made by each procedure was measured on 1.5% (by mass) agarose gels. In both cases the cDNA averaged over 2.8 kilobase pairs (lane *a*, Land *et al.*; lane *b*, Wickens *et al.*), with both containing cDNA corresponding to the upper molecular mass limit of the RNA used to direct first strand synthesis. ds cDNA was more than 95% S_1-resistant (lanes *c* and *d*). ^{32}P-labelled *Hin*d III linkers were blunt-end ligated to the cDNA shown in lane *b*. The cDNA was digested with *Hin*d III, separated from the linkers, and ligated into phosphatase-treated *Hin*d III digested pBR 322. The numbers at the left are kilobase pairs.

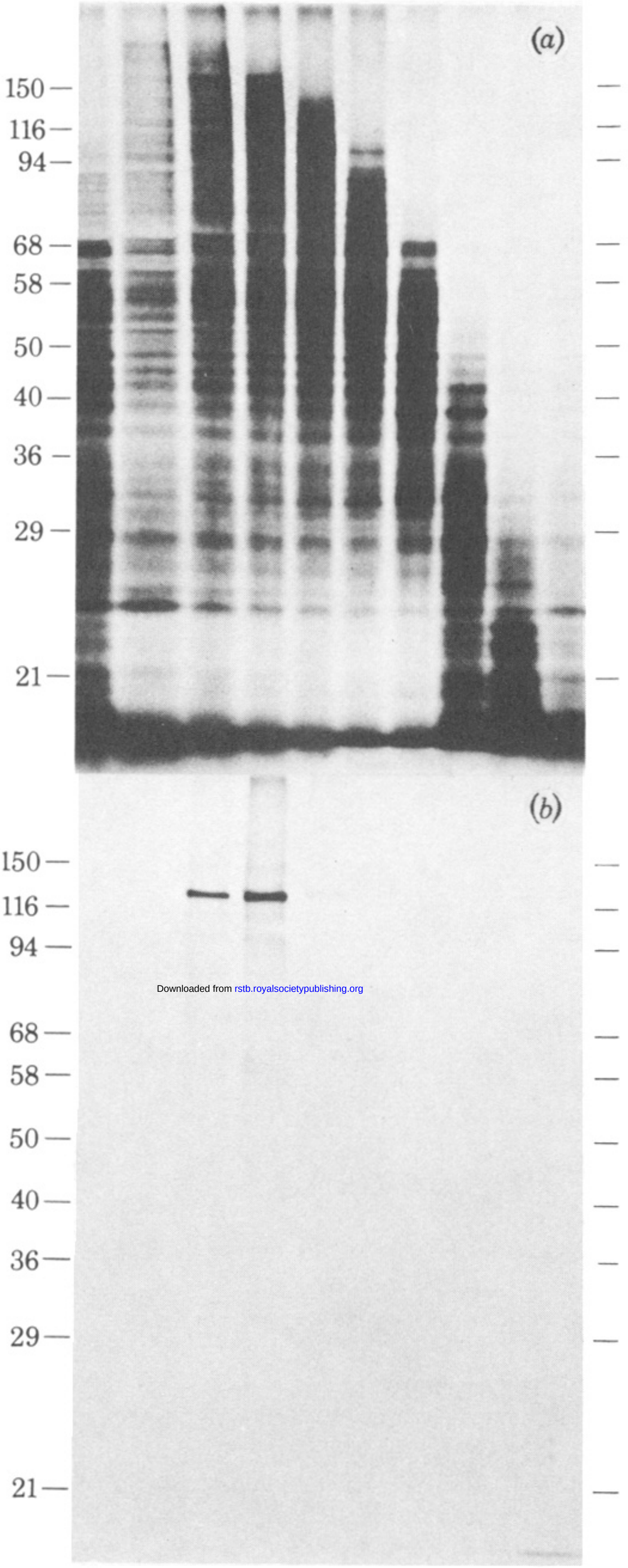


FIGURE 7. Sucrose gradient (50–300 g l^{-1}) fractionation of polysomal poly(A) RNA from etiolated, unirradiated *Avena* shoots. (*a*) Total translation products from each fraction. (*b*) Immunoprecipitates prepared from each fraction with antiphytochrome IgG. Phytochrome mRNA, concentrated in fractions 3 and 4, is enriched 10–20-fold relative to initial unfractionated poly(A) RNA.

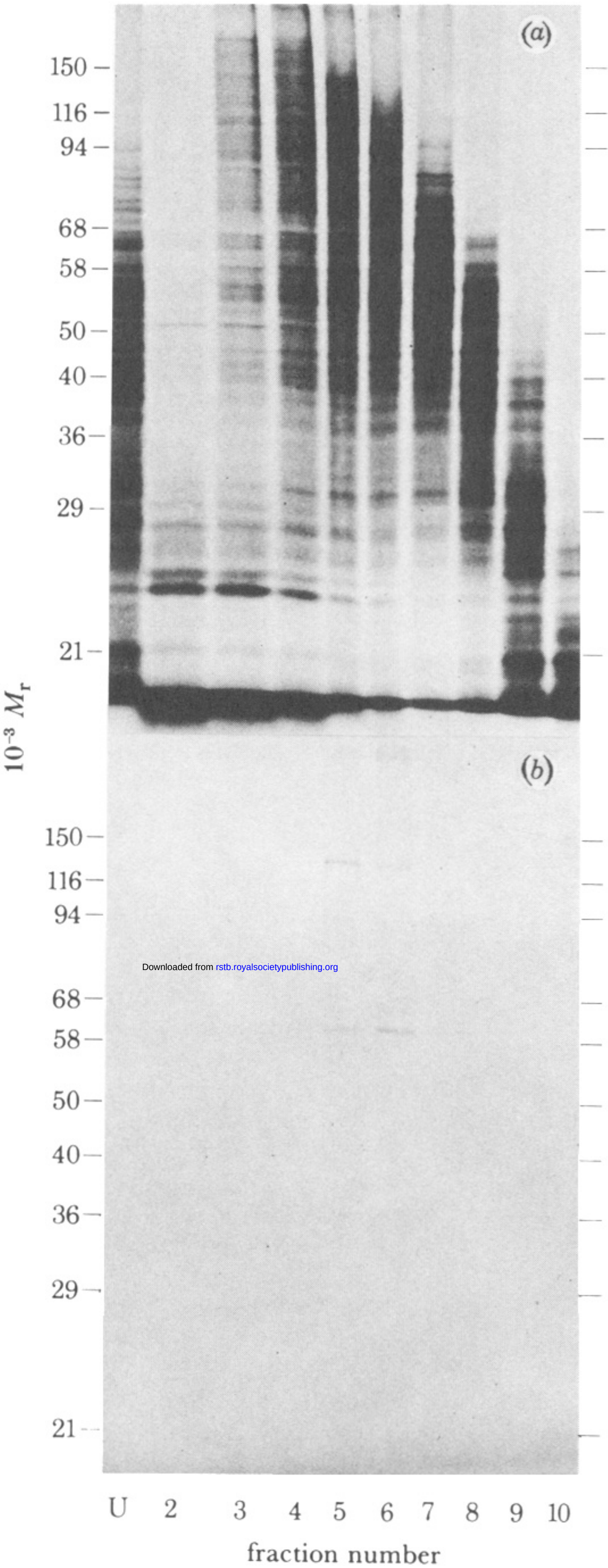


FIGURE 8. Sucrose gradient (50–300 g l^{-1}) fractionation of polysomal poly(A) RNA from etiolated oats irradiated with 5 min of red light and returned to the dark for a further 3 h before harvest. (*a*) Total translation products from each fraction. (*b*) Immunoprecipitates prepared from each fraction with antiphytochrome IgG. Phytochrome mRNA is in fractions 5 and 6 at a considerably lower level than in comparable fractions from unirradiated tissue (figure 7).