Plant Physiol. (1984) 74, 123–127
0032-0889/84/74/0123/05/$01.00/0

Characterization by Enzyme-Linked Immunosorbent Assay of Monoclonal Antibodies to *Pisum* and *Avena* Phytochrome[1]

Received for publication May 31, 1983 and in revised form August 8, 1983

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ABSTRACT

Nine monoclonal antibodies to pea (*Pisum sativum* L.) and 16 to oat (*Avena sativa* L.) phytochrome are characterized by enzyme-linked immunosorbent assay against phytochrome from six different sources: pea, zucchini (*Cucurbita pepo* L.), lettuce (*Lactuca sativa* L.), oat, rye (*Secale cereale* L.), and barley (*Hordeum vulgare* L.). All antibodies were raised against phytochrome with a monomer size near 120,000 daltons. Nevertheless, none of them discriminated qualitatively between 118/114-kilodalton oat phytochrome and a photoreversible, 60-kilodalton proteolytic degradation product derived from it. In addition, none of the 23 antibodies tested discriminated substantially between phytochrome—red-absorbing form and phytochrome—far red-absorbing form. Two antibodies to pea and six to oat phytochrome also bound strongly to phytochrome from the other species, even though these two plants are evolutionarily widely divergent. Of these eight antibodies, two bound significantly to all of the six phytochrome preparations tested, indicating that these two may recognize highly conserved regions of the chromoprotein. Since the molecular function of phytochrome is unknown, these two antibodies may serve as unique probes for regions of this pigment that are important to its mode of action.

Plant growth and development is influenced strongly by both the quantity and quality of incident light. Such effects of light are often mediated by the chromoprotein phytochrome. The molecular mechanism by which phytochrome performs its function is unknown in spite of intensive study over an extended period of time (14, 16). To a large extent, the dearth of information concerning the molecular activity or activities of this chromoprotein derive from the relatively limited scope of assays available for its study. In particular, the most widely utilized assays have been those deriving from its unique, photointerconvertible properties (1). These assays suffer both from being relatively insensitive and from being unusable in the presence of significant amounts of Chl (18). Phytochrome is, however, a good antigen (8, 15, 22). Immunochemical assays can thus be used to supplement information about the biochemical and biophysical properties of this pigment that is obtained by spectrophotometric means. Moreover, the recent development of technology for the production of monoclonal antibodies (13), as well as the recent application of this technology to phytochrome (4), greatly enhance the potential scope of immunochemical assays.

Monoclonal antibodies can, of course, be used for relatively standard applications such as immunohistochemical localization of phytochrome (24), and phytochrome quantitation by radioimmunoassay or ELISA[2] (25), as can be more conventionally obtained polyclonal antisera (9, 20). Of greater interest, however, are applications for monoclonal antibodies that cannot be satisfied by use of polyclonal antisera. With respect to phytochrome, these applications include a search for antibodies specific to the Pr or Pfr form. Such antibodies would, for the first time, permit both independent immunolocalization of the two forms as well as the possibility for their independent quantitation in green plants. Additionally, monoclonal antibodies can uniquely serve as structural probes of the phytochrome molecule. For example, by searching for those antibodies that bind to this pigment regardless of its source, it should be possible to identify domains on the chromoprotein that have been conserved through evolution. While not necessarily the case, one or more such domains might be related to its molecular function. We report here an initial characterization of 25 monoclonal antibodies to phytochrome, with an emphasis on searching for antibodies specific for one of the two forms of the pigment and for antibodies that recognize epitopes (= antigenic determinants) found on phytochrome regardless of its source.

MATERIALS AND METHODS

Phytochrome Preparations. Phytochrome was purified from six different etiolated plant tissues: oat(*Avena sativa* L. cv Garry), barley (*Hordeum vulgare* L. cv Harrison), rye (*Secale cereale* L. cv Balbo), pea (*Pisum sativum* L. cv Alaska), zucchini (*Cucurbita pepo* L. cv Black Beauty), and lettuce (*Lactuca sativa* L. cv Grand Rapids). Plant growth and storage prior to extraction were as described previously (3, 15). The purity of a phytochrome sample is estimated by its SAR. One unit of phytochrome, which is about 1.2 mg for oat phytochrome with 118/114-kD monomers (23), is the quantity that, when dissolved in 1.0 ml, has an A_{667} = 1.0 for a 1-cm optical path. Both 118/114-kD and 60-kD oat phytochrome represent proteolytic degradation products of the native molecule (12, 17, 26). Phytochrome of 118/114 kD has lost 60 to 100 amino acids, while 60-kD phytochrome is only about one-half the native monomer size. Nevertheless, both sizes of phytochrome exhibit photoreversibility similar, although not identical, to that of the native molecule (21, 27). The other phytochrome preparations, consisting of approximately 120-kD

[1] This research was supported by grants from the United States National Science Foundation (PCM-8022159), the United States Department of Energy (DE-AC09-81SR10925), and the Fonds Marc Birkigt, Geneva, Switzerland.

[2] Abbreviations: ELISA, enzyme-linked immunosorbent assay; SAR, specific absorbance ratio, A_{667}/A_{280} with phytochrome as Pr; IgG, immunoglobulin G.

monomers, are also likely degraded proteolytically to about the same extent as 118/114-kD oat phytochrome, but this possibility has not been rigorously tested.

Oat phytochrome of 118/114 kD was purified to near homogeneity (SAR = 0.77; see Fig. 1 in Ref. 4 for SDS PAGE profile of this sample) by immunoaffinity chromatography, with elution from the antibody column induced by 3 M $MgCl_2$ (10). Oat phytochrome of 60 kD was purified to virtual homogeneity (SAR = 1.10; see Fig. 7 in Ref. 16 for SDS PAGE profile of a comparable sample) by sequential chromatography with brushite, carboxymethyl-Sephadex, DEAE-cellulose, brushite, and Sephadex G-200 columns (9). Pea phytochrome of about 120 kD was purified to approximately 40% homogeneity (SAR = 0.23 [2]) by sequential chromatography through brushite, DEAE-Bio-Gel A, brushite, and Sephacryl S-300 columns (9, 19). Zucchini phytochrome of about 120 kD was purified to near homogeneity (SAR = 0.54; see Fig. 4 in Ref. 2 for SDS PAGE profile of a comparable sample) by immunoaffinity chromatography, with elution from the antibody column induced by 3 M $MgCl_2$, pH 7.8 (2). Lettuce phytochrome, which presumably included degradation products in addition to the approximately 120-kD size of the polypeptide, was immunopurified as for zucchini phytochrome (SAR = 0.19; see Fig. 5b in Ref. 2 for SDS PAGE profile of a comparable preparation). Barley (SAR = 0.1) and rye (SAR = 0.2) phytochrome were both partially purified by sequential chromatography through brushite, DEAE-cellulose and Bio-Gel P-300 columns as described before (15). Prior to use, phytochrome preparations were stored at or below −70°C in either 60 mM K-phosphate, pH 7.8 (rye, barley, and 60-kD oat phytochrome), or 0.1 M Na-phosphate, 1 mM EDTA, pH 7.8 (118/114-kD oat, and approximately 120-kD pea, zucchini, and lettuce phytochrome).

Monoclonal Antibodies. Preparation and initial identification of the monoclonal antibodies used here are described in detail elsewhere (4). Antibody-secreting cells were obtained from mice immunized with either approximately 120-kD pea or 118/114-kD oat phytochrome. Assays to screen hybridomas for the secretion of antibodies to phytochrome were done under white light so that there would be no selection at this stage for antibodies to only one of the two forms. These screening assays utilized goat or rabbit antibodies to mouse IgG as second antibody. Thus, only monoclonal antibodies recognized by antibodies to IgG would be detected. Antibody-containing hybridoma medium was concentrated 20-fold by precipitation with half-saturation $(NH_4)_2SO_4$ (4). Monoclonal antibodies were tested either as present in this $(NH_4)_2SO_4$-concentrated medium or after immunopurification with a column of immobilized rabbit antibodies to mouse IgG (4).

ELISA. ELISAs were performed in 96-well vinyl plates by modification of the protocol used for initial screening (4). In principle, this protocol involves (a) nonspecific adsorption of phytochrome to the assay wells, followed by (b) blocking with BSA, which minimizes further nonspecific adsorption of proteins to the assay well, (c) addition of monoclonal antibody, (d) addition of alkaline phosphatase-conjugated goat antibody to mouse IgG, and (e) color development following addition of *p*-nitrophenyl phosphate as substrate for alkaline phosphatase. Modifications from its earlier use (4) are: (a) incubation of phytochrome for 2 h at 4°C rather than at room temperature in order to minimize possible proteolysis, and (b) termination of enzyme reactions by addition of 50 µl of 3 N NaOH and measurement of absorbance values at 410 nm with a Minireader MR590 (Dynatech, Alexandria, VA). Except when comparing activity between Pr and Pfr, assays were done under white fluorescent room light.

To compare monoclonal antibody binding to Pr and Pfr the following additional modifications were included. (a) All work prior to the addition of substrate solution was done under green light that was not significantly absorbed by either form of phytochrome (15). (b) After dilution to the highest concentration tested, but before addition to the assay wells, phytochrome samples were converted to Pr by saturating irradiation with a 45-w microscope lamp (Unitron LKR, Newton Highlands, MA) after its output was filtered through a 737-nm interference filter (type B-40, Balzers, Liechtenstein). Samples were then diluted and phytochrome was added to the plates as Pr. The remainder of each dilution was then given a saturating irradiation as before, but this time with a 663-nm filter (type B-40, Balzers) to convert phytochrome to Pfr, after which Pfr was added to the wells. (c) Wash solution was kept at 4°C and incubations with blocking solution and monoclonal antibody were also kept at 4°C to minimize the extent of any thermal reversion of Pfr to Pr that might occur.

RESULTS

All 25 monoclonal antibodies, 9 of which were raised against pea phytochrome and 16 of which were raised against oat phytochrome (4), were tested qualitatively for activity by ELISA against phytochrome from six different plant species and against both 118/114-kD and 60-kD oat phytochrome (Table I). While each antibody, with a few exceptions (notably oat-18 and oat-21), reacted well with its original antigen, the pattern of activity with respect to phytochrome isolated from other species was highly variable. Some antibodies reacted well with phytochrome regardless of source (*e.g.* oat-12 and oat-20), some only with monocotyledonous phytochrome (*e.g.* oat-8 and oat-22), some only with dicotyledonous phytochrome (*e.g.* I-13b6 and I-15a3), and some only with the original antigen used for its production (*e.g.* I-3b2 and oat-4). A few exhibited high background (*e.g.* oat-1 and oat-7), but in every case this background activity was many-fold lower than that seen when the assay wells were coated with phytochrome.

Comparison of Reactivity to Pr and Pfr. All but two of the monoclonal antibodies (oat-18 and oat-21, both of which reacted very weakly to either Pr or Pfr [Table I]) were compared by ELISA with respect to their ability to discriminate between the two forms of phytochrome. The results of only four such assays are presented as examples, although all 23 were assayed identically (Fig. 1). The most extreme difference in reactivity for the monoclonal antibodies to pea phytochrome was I-3b2, while the greatest similarity was exhibited by I-15a3. The small differences observed, since they were evident with all antibodies to pea phytochrome, probably reflect differential binding of pea phytochrome in its two forms to the vinyl assay wells. For monoclonal antibodies to oat phytochrome, the most extreme difference was exhibited by oat-7, the greatest similarity by oat-13. Results obtained with oat-13 are also typical of those obtained with the remaining monoclonal antibodies to oat phytochrome. Since identical results were obtained for all 23 antibodies in two independent experiments, it appears that the difference observed with oat-7 is quantitatively significant. Nevertheless, the difference is small.

Comparison of Reactivity to 118/114-kD and 60-kD Oat Phytochrome. When the 18 monoclonal antibodies that reacted well with oat phytochrome were compared with respect to their ability to bind to 118/114-kD and 60-kD sizes of oat phytochrome, it was observed that all bound well to both sizes, although some quantitative differences were observed (Table I; Fig. 2). Data comparable to those in Figure 2 were obtained for I-11a12, I-18a1, II-18a4, II-19a1, oat-3, oat-4, oat-7, oat-11, oat-12, oat-13, oat-14, oat-16, oat-20, and oat-22 but are not shown. The difference in reactivity observed with oat-8 is the extreme example, whereas the data for oat-9 are typical. Data obtained with oat-17 represent the most similar activity that was observed.

Table I. *Screening of 25 Monoclonal Antibodies to Phytochrome against Seven Different Phytochrome Preparations*

Results are presented as normalized absorbance values at 410 nm. Each entry represents the mean of three values from two independent experiments. For each experiment, the highest absorbance value (1.94 or 2.14) was set arbitrarily to 100 to facilitate evaluation of the results.

Monoclonal Antibody	Phytochrome[a]							BSA[b]
	Zucchini	Lettuce	Pea	Oat	Oat	Rye	Barley	
				118/114 kD	*60 kD*			
To pea phytochrome								
I-3b2[c]	2	2	73	3	3	2	3	1
I-9a2[c]	10	5	62	8	7	5	5	1
I-11a4[d]	60	44	56	66	49	3	3	1
I-11a12[c]	45	51	74	18	16	27	24	5
I-13b6[c]	12	2	67	3	3	3	3	1
I-15a3[c]	14	3	77	3	3	2	2	1
I-18a1[c]	75	30	81	16	13	34	29	1
II-18a4[c]	77	38	77	29	17	30	24	1
II-19a1[d]	48	36	50	51	47	17	8	1
To oat phytochrome								
Oat-1[d]	32	29	31	42	33	31	37	9
Oat-3[c]	6	12	17	76	69	46	16	1
Oat-4[d]	2	2	3	66	38	18	4	2
Oat-7[d]	41	20	39	45	34	25	30	6
Oat-8[c]	14	14	14	98	90	87	69	1
Oat-9[c]	51	24	43	94	89	78	62	1
Oat-11[c]	37	25	30	80	67	55	45	3
Oat-12[d]	68	50	62	65	63	54	57	7
Oat-13[c]	14	11	37	95	92	80	49	1
Oat-14[e]	11	10	10	41	35	13	11	5
Oat-16[c]	37	8	25	91	79	78	58	1
Oat-17[e]	24	38	26	47	47	13	6	2
Oat-18[d]	14	12	15	16	15	11	13	1
Oat-20[d]	50	28	47	48	55	50	60	2
Oat-21[c]	12	2	2	16	13	7	5	1
Oat-22[c]	7	5	6	100	92	84	67	1
Nonimmune mouse IgG[f]	4	3	3	4	3	2	3	1
Minus antibody[g]	2	2	2	2	2	2	3	1

[a] Assay wells were coated with phytochrome at 5 munits/ml.
[b] Control wells were coated with BSA at 6 μg/ml in borate-saline buffer.
[c] Monoclonal IgG was immunopurified and used at a concentration of 10 μg/ml.
[d] Monoclonal IgG was assayed in $(NH_4)_2SO_4$-concentrated medium after 10-fold dilution.
[e] Monoclonal IgG was assayed in undiluted $(NH_4)_2SO_4$-concentrated medium.
[f] Nonimmune mouse IgG was used at 10 μg/ml in place of monoclonal IgG.
[g] Only diluent was added to assay wells in place of monoclonal IgG.

Quantitation of Cross-Reactivity against Monocotyledonous and Dicotyledonous Phytochrome. The eight monoclonal antibodies that exhibited the greatest cross-reactivity when comparing monocotyledonous to dicotyledonous phytochrome (Table I) were compared quantitatively by testing against different concentrations of phytochrome (Fig. 3). Whle oat-1 and oat-7 exhibited approximately equivalent reactivity to both oat and pea phytochrome, I-11a4, II-19a1, oat-9, and oat-11 each reacted better with its original antigen. Oat-12 and oat-20 were tested with all six phytochrome preparations because they exhibited in qualitative assays (Table I) the greatest extent of cross-reactivity. Both exhibited approximately equivalent reactivity against each phytochrome preparation except that from barley.

DISCUSSION

Prior to evaluation of the data presented here, a general note of caution is advisable. Since the relationship between phytochrome units, as used here, and absolute phytochrome quantities is unknown, and since the different phytochrome preparations (Table I; Fig. 3), sizes (Table I; Fig. 2), or forms (Fig. 1) may exhibit differential binding to the vinyl assay wells, absolute quantitative comparisons cannot readily be made. Thus, differences in activity for a single monoclonal antibody when tested against different phytochrome preparations (*e.g.* oat-12 in Table I and Fig. 3) might result from differential binding of the phytochrome samples to the vinyl assay wells rather than from different affinities of the antibody for the different phytochromes. Nevertheless, comparative evaluations of the data are possible. For example, it is clear that oat-7 discriminates very slightly between Pr and Pfr since the other monoclonal antibodies (*e.g.* oat-13) show superimposable activity with respect to the two forms (Fig. 1), which implies that they bind equally well to the assay wells. The alternative interpretation, which is that they do not bind equally well and that oat-7 does not discriminate between them while the other 13 monoclonal antibodies to oat phytochrome do, is sufficiently implausible that it may be discarded. These inherent limitations in interpretation of the data should be kept in mind during the discussion that follows.

Comparative immunochemistry of phytochrome using polyclonal rabbit antisera or polyclonal antibodies immunopurified

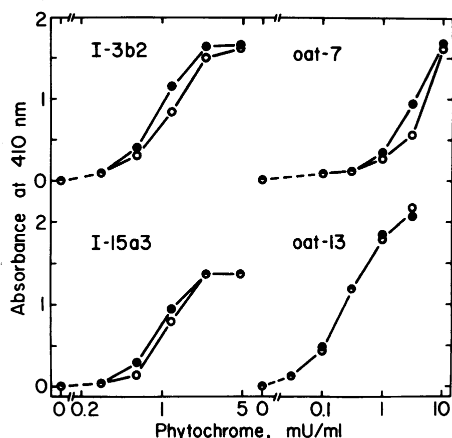

FIG. 1. Examples of the relative ELISA activity of the monoclonal antibodies when tested against the two different forms of phytochrome. Vinyl assay plate wells were coated with the indicated concentrations (mU = milliunit) of pea (I-3b2, I-15a3) or 118/114-kD oat (oat-7, oat-13) phytochrome as either Pr (●) or Pfr (○).

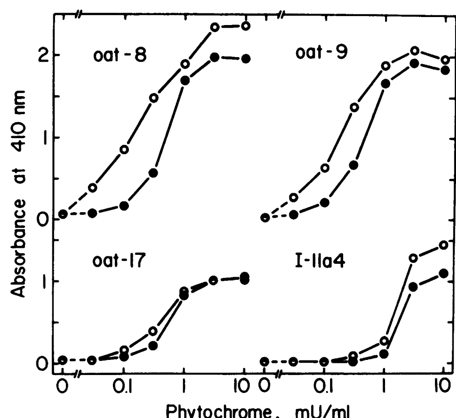

FIG. 2. Examples of the relative ELISA activity of the monoclonal antibodies when tested against 118/114-kD and 60-kD oat phytochrome. Vinyl assay plate wells were coated with the indicated concentrations (mU = milliunit) of either 118/114-kD (○) or 60-kD (●) oat phytochrome.

from these sera indicated previously that antibodies raised to phytochrome from a monocotyledonous plant (oat) cross-react well with phytochrome from other monocotyledons but only weakly against phytochrome from dicotyledons (6, 15, 22). Conversely, antibodies to phytochrome from a dicotyledonous plant (zucchini, pea, or lettuce) cross-react well with phytochrome from other dicotyledons but poorly with phytochrome from monocotyledons (3). Moreover, micro-complement fixation assays have indicated that pea phytochrome possesses epitopes that are absent in oat phytochrome (15). Data presented here (Table I; Fig. 3) are consistent with these earlier observations and indicate further that by appropriate selection one may choose an antibody that either (a) is highly specific for the phytochrome used as antigen (*e.g.* I-3b2), (b) exhibits a wide range of cross-reactivity and may, therefore, be useful for work with phytochrome from a large number of evolutionarily divergent plant species (*e.g.* oat-12), (c) is highly specific for phytochrome from monocotyledons as opposed to dicotyledons (*e.g.* oat-22), or (d) is specific for phytochrome from dicotyledons as opposed to monocotyledons, although the specificity presently available (I-11a12) may not be sufficient for all purposes. These highly variable patterns and extents of cross-reactivity will permit appropriate selection in the future of specific monoclonal antibodies that have the highest probability of being useful for predetermined applications. These same data also indicate that these antibodies are, in fact, either recognizing a relatively large number of different epitopes on phytochrome, which is not surprising since it is a globular protein with a large polypeptide monomer (124 kD from oat [12, 26]), or that, as a minimum, they recognize a smaller set of epitopes but in variable ways.

One of the most important potential applications to phytochrome of monoclonal antibodies would be the development of one or more antibodies specific to the inactive Pr or the active Pfr conformation. The immunization protocol used for the production of the antibodies characterized here was not designed specifically for this purpose, although initial screening of hybridomas was done so that if such an antibody were obtained it would have been detected (4). Nevertheless, none of the 23 antibodies tested for this property discriminated substantially between the two forms by the ELISA used here (Fig. 1, only extreme data shown). Since Pr and Pfr concentrations were varied over a wide range to ensure that antigen would be limiting, it is evident that there was not even a marked quantitative difference in the ability of the antibodies to discriminate between the two. Since the number of antibodies tested here is relatively large, and since all but one attempt (8) to discriminate between Pr and Pfr with polyclonal rabbit antisera have failed (22), even when the sensitive micro-complement fixation assay was used (6, 15), it appears that it will be necessary to immunize mice with, for example, phytochrome that has been stabilized by internal cross-linking with a bifunctional reagent to maximize the probability that selective antibodies will be obtained. Alter-

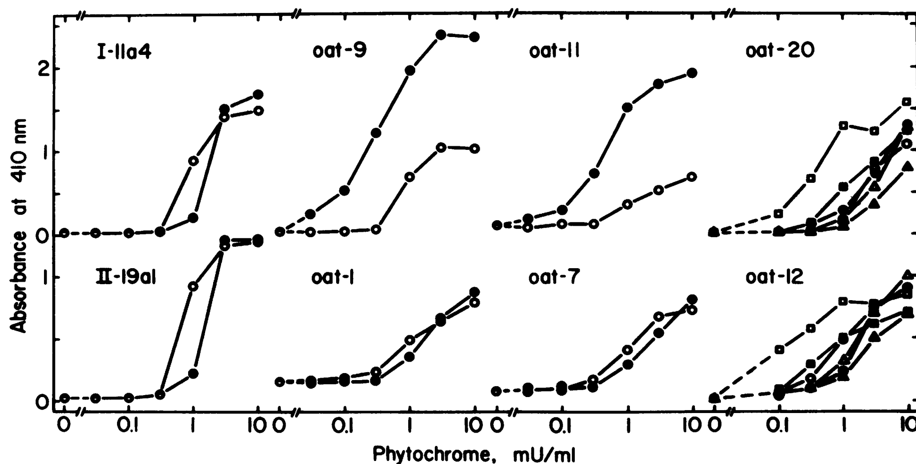

FIG. 3. Cross-reactivity of eight monoclonal antibodies to phytochrome against phytochrome from different plants as indicated by ELISA activity. The indicated monoclonal antibodies were tested against different concentrations (mU = milliunit) of 118/114-kD oat (●), pea (○), lettuce (▲), zucchini (△), rye (■), or barley (□) phytochrome.

natively, it is possible that for some unanticipated reason, thermal reversion of Pfr to Pr is catalyzed by interaction with the vinyl surface of the assay well, thus possibly accounting for the failure to detect an antibody specific to one form of the pigment. A different assay approach must be developed to test for this possibility.

Polyclonal rabbit antisera raised against 118/114-kD oat phytochrome contain immunoglobulins that bind to epitopes on 118/114-kD phytochrome that are missing from 60-kD phytochrome, as indicated not only by Ouchterlony double immunodiffusion but also by common antigen immunoelectrophoresis (5) and micro-complement fixation assay (6). Furthermore, these polyclonal antisera bind to proteolytic degradation fragments derived from 118/114-kD phytochrome that antisera to 60-kD oat phytochrome do not recognize (7). One would, therefore, expect in a sample of monoclonal antibodies to oat phytochrome as large as that studied here (see Fig. 2 for examples), that some would exhibit specificity for 118/114-kD as opposed to 60-kD oat phytochrome. It is surprising that none do. It is not known whether this negative outcome is the result of chance, selective recognition by the immunized mice of epitopes on 60-kD phytochrome, or selection for antibodies to 60-kD phytochrome during the initial screening process. Since the initial screening was done with immunopurified, 118/114-kD phytochrome (4), which had earlier been shown to be free of protease activity (11), it appears unlikely that the reason for this outcome is because of selection at this stage. Nevertheless, in future screening of new hybridomas, it will be important to pay attention to the possibility of protease activity in the hybridoma medium as it is tested for the presence of antibody to phytochrome. Such antibodies specific for epitopes not present on 60-kD phytochrome will be important for the purification and characterization of substantial fragments of the phytochrome molecule that do not include chromophore and, therefore, cannot be detected in any other way (7).

A search for antibodies that recognize an epitope found on phytochrome regardless of its source has, by contrast, been fruitful. Two such monoclonal antibodies have been identified, at least within the limits probed here (Fig. 3; oat-12, oat-20). Further characterization of this (these) cognate antigenic region(s) of phytochrome, to which these two antibodies bind, may lead to important new information about the molecule as outlined in the Introduction. It will also be of interest to determine whether these antibodies bind to phytochrome from organisms such as *Mougeotia*, which have yielded significant information concerning phytochrome function from physiological studies (14), but from which it has been virtually impossible to purify sufficient phytochrome for raising antibodies or for independent biochemical and biophysical analyses.

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