Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 734–738, January 1995
Plant Biology

Nuclear localization signal binding proteins in higher plant nuclei

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Communicated by Anton Lang, Michigan State University, East Lansing, MI, October 12, 1994 (received for review August 31, 1994)

ABSTRACT **The import of proteins into the nucleus is a vital process that is mediated by proteins which specifically recognize nuclear localization signals (NLSs). These factors have not been identified in plants. Previously, we demonstrated that higher plants possess a low-affinity binding site at the nuclear pore that specifically binds to several classes of functional NLSs. By the use of crosslinking reagents and a radiolabeled peptide to the bipartite NLS from the endogenous plant transcription factor Opaque2, two NLS binding proteins (NBPs) of 50–60 kDa and at least two NBPs of 30–40 kDa were identified. Competition studies indicated that labeling was specific for the functional NLS but not a mutant NLS impaired *in vivo* or a peptide unrelated to NLSs. Also, the apparent dissociation constant (100–300 μM) for labeling was similar to that of the binding site. Proteins of similar mass were labeled with two different crosslinking reagents, and concentration and time studies indicated that these NBPs were distinct proteins and not aggregates. Treatment with salt, detergent, or urea before or during NLS binding demonstrated that the properties of the binding site and the NBPs were identical. This tight correlation strongly indicates that some or all of the NBPs constitute the nuclear pore binding site. Overall, our results indicate that some components of NLS recognition are located at the nuclear pores in higher plants.**

The movement of macromolecules across the nuclear envelope (NE) is an essential process and occurs through nuclear pore complexes (NPCs; reviewed in ref. 1). The import of proteins into the nucleus is mediated by nuclear localization signals (NLSs), most of which can be categorized into one of three classes (reviewed in refs. 2 and 3). The simian virus 40 (SV40) large T-antigen NLS (4, 5) is the most thoroughly studied signal and typifies a class of NLSs possessing a single basic amino acid domain. Another class of NLSs known as bipartite signals (6) is composed of two basic regions separated by a spacer. Finally, the Matα2-type NLSs (7) possess hydrophobic and basic amino acids.

In yeast and animals, nuclear import involves energy-independent NLS binding at the NPC followed by translocation, which requires ATP hydrolysis (reviewed in ref. 1). Plants may also require ATP hydrolysis for import (8). Studies in mammals, however, indicate that GTP hydrolysis may at least indirectly influence nuclear import (reviewed in ref. 9), although there is evidence to the contrary (10). Because import is saturable and specific (11), it is thought to be receptor mediated. Several approaches have been used to identify NLS binding proteins (NBPs) in yeast and animals, including genetic (12, 13) and biochemical (reviewed in ref. 1) strategies.

In plants, little is known about nuclear targeting (reviewed in ref. 3). A number of studies have focused on NLSs from plant viruses (14) and bacteria (reviewed in ref. 15). In addition, several reports indicate that the SV40 large T-antigen NLS functions in plants (16–18), and a specific mutation known to impair NLS function in mammals (4, 5) also impairs function in plants (17, 18). We have examined the nuclear import of several plant transcription factors (19, 20) including Opaque2 (O2; ref. 20) that possesses SV40-like and bipartite NLSs that are functional *in vivo* (21). Mutations were also introduced into the bipartite NLS that impair its ability to direct import (18).

In contrast to NLSs, almost nothing is known about the import apparatus in plants. In particular, components that specifically recognize NLSs are poorly defined. To explore this step of import, we examined the binding of two classes of NLSs to purified tobacco and maize nuclei. Both ^{14}C- and ^{125}I-labeled peptides to the bipartite NLS from O2 and the SV40 large T-antigen NLS were found to bind to and compete for a single low-affinity site that is proteinaceous and firmly associated with the NE and NPCs (22). As an important intermediate step in understanding the import apparatus of plants, we have used crosslinking reagents under conditions similar to those used for binding. We have identified several polypeptides that bind specifically to functional NLSs, and their affinities and biochemical properties tightly correlate with the previously characterized binding site. These results indicate that in plants some components of NLS recognition are located at the NPC.

MATERIALS AND METHODS

Materials and Purification of Nuclei. Chemicals were obtained from Sigma unless noted otherwise. Synthetic peptides, which were described (22), had the following sequences: functional O2 bipartite NLS (O2WT), MPTEERVRKR KESNRESARR SRYRKAAHLK C; mutant O2 NLS (O2Mut), MPTEERVRTN KESNRESARR SNYR-KAAHLK C; peptide unrelated to NLSs, CDGVFAGGG. The peptide unrelated to NLSs corresponds to a signal from barley lectin that is defective in specifying vacuolar targeting (23). Nuclei were prepared from *Nicotiana tabacum* cells by the abbreviated protocol described (22) except that dithiothreitol was omitted from the nuclei isolation buffer.

Specific Labeling of NBPs. The O2WT peptide was ^{14}C- or ^{125}I-labeled as described (22). Briefly, for ^{14}C labeling, peptide was allowed to react with 50 μCi of iodo[^{14}C]acetamide (21.1 mCi/mmol; 1 Ci = 37 GBq; Amersham). After 4 hr, unreacted iodo[^{14}C]acetamide was removed by gel filtration, and [^{14}C]O2WT was concentrated by lyophilization. For ^{125}I labeling, Iodo-Gen (Pierce) was used according to the manufacturer. To identify NBPs, 1×10^6 tobacco nuclei were diluted to 50 μl with labeling buffer [50 mM Hepes, pH 7.8/25 mM KCl/2.5 mM $MgCl_2$/3 mM $CaCl_2$/20% (vol/vol) glycerol] and 10 units of DNase I (Boehringer Mannheim) was added. After a 15-min incubation at room temperature, the nuclei were centrifuged at 12,000 × *g* for 2 min, and the pellet was suspended in 30 μl of ice-cold labeling buffer containing 80,000–100,000 cpm of [^{14}C]O2WT (final concentration, 10–20 μM) or, where noted, $1–1.5 \times 10^6$ cpm of [^{125}I]O2WT

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Abbreviations: DSS, disuccinimidyl suberate; NBP, nuclear localization signal binding protein; NE, nuclear envelope; NLS, nuclear localization signal; NPC, nuclear pore complex; SV40, simian virus 40; MBS, maleimidobenzoyl *N*-hydroxysuccinimide ester.

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(final concentration, 0.2–0.4 μM). The radiolabeled peptide was allowed to associate with the nuclei for 5 min on ice, at which time the crosslinking reagents disuccinimidyl suberate (DSS; Pierce) in dimethyl sulfoxide or maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS; Pierce) in dimethylformamide was added to a final concentration of 5 mM. The concentration of dimethyl sulfoxide or dimethylformamide never exceeded 10% (vol/vol) of the final reaction volume. Crosslinking was allowed to proceed for 10 min at room temperature. After centrifugation at 12,000 × *g* for 2 min, the nuclear pellet was suspended in 25 μl of SDS/PAGE loading buffer (containing Tris and dithiothreitol) to quench the unreacted crosslinking reagent, and the proteins were subjected to electrophoresis through a SDS/10% polyacrylamide gel. Gels were stained with Coomassie blue R-250, treated with a fluorographic enhancer (Fluorohance, Research Products International), and dried. Radiolabeled NBPs were visualized by fluorography at −80°C for 5–12 days. This is referred to as the standard labeling reaction.

For competition experiments, peptides were added to the standard labeling reaction mixture from concentrated stocks made in labeling buffer. To examine the possibility that competitor peptides could scavenge unreacted DSS, [^{14}C]O2WT was allowed to bind to nuclei in the presence of unlabeled O2WT, O2Mut, or a peptide unrelated to NLSs at final concentrations of 700 μM. The nuclei were then pelleted to eliminate unbound competitor peptides. After suspension in 30 μl of labeling buffer, crosslinking was performed as for the standard labeling reaction. For DSS concentration experiments, the reagent was added to the standard labeling reaction mixture to the final concentrations indicated. To optimize the time of labeling, the standard labeling reaction was used except that the reaction time was as indicated. For pH experiments, the standard labeling reaction was used except where appropriate the 50 mM Hepes (pH 7.8) in the labeling buffer was replaced by the following buffers: 50 mM 2-(*N*-morpholino)ethanesulfonic acid KOH (pH 5.7), 50 mM Hepes (pH 6.5), 50 mM Hepes (pH 7.3), or 50 mM $NaHCO_3$ (pH 9.0).

For extraction of nuclei before binding, DNase I-treated nuclei were suspended in 30 μl of labeling buffer or labeling buffer plus 0.25 M NaCl, 1% (vol/vol) Triton X-100, or 6 M urea. After 15 min on ice, the nuclei were pelleted, suspended in labeling buffer with [^{14}C]O2WT, and processed as for the standard labeling reaction. For disruption of binding, DNase I-treated nuclei were suspended in 29 μl of labeling buffer or labeling buffer with the reagents indicated above. After 15 min on ice, [^{14}C]O2WT peptide was added in 1 μl, and binding was allowed to proceed for 5 min on ice. The DSS was then added and the samples were processed as for the standard labeling reaction. All crosslinking experiments were done at least twice with different nuclear preparations except for competition and extraction/disruption experiments, which were done at least four times.

Nuclear Binding Assays. To compare the properties of the NLS binding site to those of the NBPs, the binding assay (22) was modified to match the standard labeling reaction. The final volume was reduced to 30 μl and the binding buffer was replaced with labeling buffer or labeling buffer with the reagents indicated above. For both the extraction and disruption experiments, DNase I-treated nuclei were processed as described for the matching crosslinking experiments except that after binding of [^{14}C]O2WT the nuclei were pelleted and suspended in 100 μl of labeling buffer; the cpm were quantitated by scintillation counting. Nonspecific background binding was estimated for all treatments by the addition of 3 mM O2WT. All assay points are averages of three experiments, with each having duplicate samples.

RESULTS

Identification of Specific NBPs. Previously (22), we developed a binding assay to detect specific NLS binding to purified plant nuclei. To identify the corresponding NBPs, a 31-amino acid peptide to the functional bipartite NLS from O2 (O2WT) was ^{14}C-labeled and allowed to bind to DNase I-treated nuclei purified from tobacco suspension cultured cells. The amine-reactive homobifunctional crosslinking reagent DSS was then added, and the resulting radiolabeled polypeptides were detected by SDS/PAGE and fluorography. Crosslinking resulted in radiolabeling of two polypeptides of 50–60 kDa and at least two polypeptides of 30–40 kDa (Fig. 1). The proteins were specifically labeled because, compared to a control with no added competitor (lane 1), the addition of increasing concentrations of O2WT during binding and crosslinking resulted in dramatically reduced labeling of the NBPs (lanes O2WT). The addition of similar concentrations of a mutated form of the O2 NLS (O2Mut), shown to be defective in stimulating import *in vivo* (18), resulted in little or no reduction in labeling (lanes O2Mut). A peptide unrelated to NLSs was also an ineffective competitor (lane 8). The lack of competition by either O2Mut or the peptide unrelated to NLSs indicates that the labeling was specific for functional NLS. The gel lanes contained equivalent quantities of protein as determined by Coomassie blue staining of the matching SDS/polyacrylamide gel (data not shown). The observed competition was not an artifact caused by a scavenging effect of the amine-containing NLS peptides for the DSS. This was demonstrated by the removal of unbound competitor before crosslinking, which reduced the specific activity of all labeling (because binding is reversible) but did not affect the results of the competition studies (data not shown). Similar results were also obtained with [^{125}I]O2WT (data not shown), although the background labeling was greater than that observed for [^{14}C]O2WT. Overall, the specificity of labeling as well as the apparent dissociation constant (K_d) for binding from competition studies (100–300 μM) was similar to the values reported for the NPC binding site (22).

Characterization of NBP Labeling. We systematically optimized conditions for crosslinking with [^{14}C]O2WT in order to gain insight into the biochemistry of the NBPs. To determine the optimum concentration of DSS for labeling, the reagent was added to concentrations as great as 10 mM (Fig. 2*A*). Although the maximum specific activity of NBP labeling was observed at a concentration of 10 mM (Fig. 2*A*), 5 mM DSS was reasoned to be optimal because this was the minimum reagent concentration examined that resulted in consistent high specific activity labeling. Minimizing the DSS concentration was relevant, since less reagent should decrease the

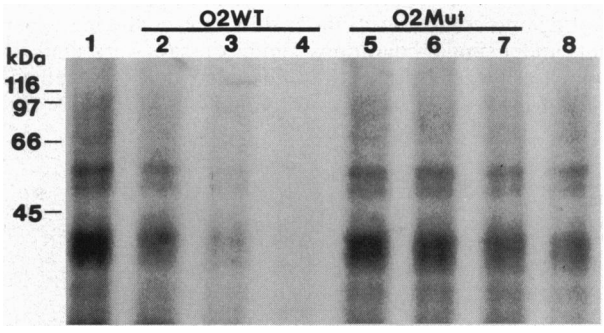

FIG. 1. Nuclear proteins from tobacco are specifically labeled with a peptide to the functional O2 NLS. Fluorograph of nuclear NBPs labeled in the absence of competitor peptide (lane 1) or in the presence of a peptide to the functional O2 NLS (lanes O2WT), an import-defective O2 mutant peptide (lanes O2Mut), or a peptide unrelated to NLSs (lane 8). Concentrations of competitor peptides are 100 μM (lanes 2 and 5), 300 μM (lanes 3 and 6), and 700 μM (lanes 4, 7, and 8). The [^{14}C]O2WT ligand was present at 10 μM.

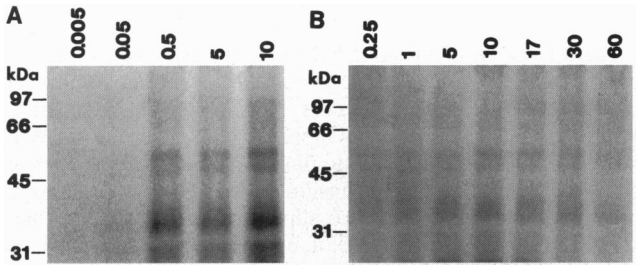

FIG. 2. Characterization of NBP labeling. (*A*) Fluorograph showing tobacco nuclear proteins labeled with increasing concentrations of the crosslinking reagent DSS for 10 min. Concentrations (mM) of DSS are indicated. (*B*) Fluorograph showing proteins labeled with 5 mM DSS with increasing reaction times. Reaction times (min) are indicated. Note for both *A* and *B* the absence of products in addition to the NBPs indicating that the NBPs are distinct polypeptides.

probability of nonspecific crosslinking events. To determine the optimum time of labeling, crosslinking was allowed to proceed for a maximum of 1 hr prior to the addition of SDS/PAGE loading buffer to inactivate unreacted crosslinking reagent (Fig. 2*B*). Ten minutes was the optimum reaction time. Longer times actually reduced the apparent labeling, indicating the formation of protein aggregates that could not be resolved by SDS/PAGE. In fact, increasing amounts of radiolabeled material that did not migrate out of the SDS/PAGE stacking gels were observed with time (data not shown). Despite the formation of these aggregates, in neither the DSS-concentration nor the reaction-time experiments were labeled proteins of greater mass than the NBPs resolved (Fig. 2). These results indicate that the labeled products that are resolved by SDS/PAGE are distinct polypeptides.

To determine the optimum pH for labeling, crosslinking with DSS was done at a broad range of pH values (Fig. 3*A*). Among the conditions tested, pH 7.8 yielded the greatest specific activity of labeling. The amine- and sulfhydryl-reactive heterobifunctional reagent MBS was also examined at the same pH values (Fig. 3*B*). Although the optimum pH for labeling with MBS (between pH 7.3 and 7.8) was somewhat different than that for DSS, both reagents labeled polypeptides of similar mass (compare Fig. 3 *A* and *B*). The polypeptides labeled with MBS were also shown to associate specifically with O2WT in competition studies (data not shown). These results indicate that NBP labeling can be achieved with several reagents of different chemical selectivities.

The NBPs Correspond to the NPC Binding Site. To examine whether some or all of the labeled NBPs constituted the previously identified binding site, binding assays were done with [^{14}C]O2WT, and the cpm were quantitated by scintillation counting and compared to the results of crosslinking experiments under similar conditions. Binding assays were similar to those described (22), except that the nuclei were treated with DNase I prior to the assay, a treatment shown to have little

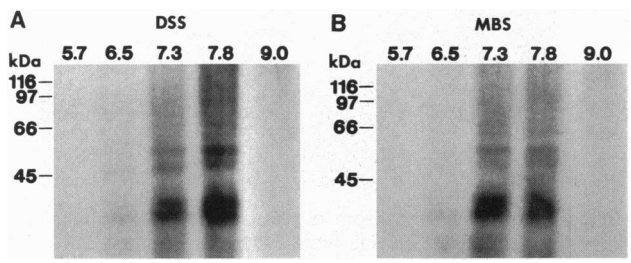

FIG. 3. Labeling of tobacco NBPs with two different crosslinking reagents. (*A*) Fluorograph of NBPs labeled with 5 mM DSS for 10 min at different pH values. (*B*) Fluorograph of NBPs labeled with 5 mM MBS for 10 min at different pH values. For both *A* and *B* the pH values of the labeling buffers are indicated.

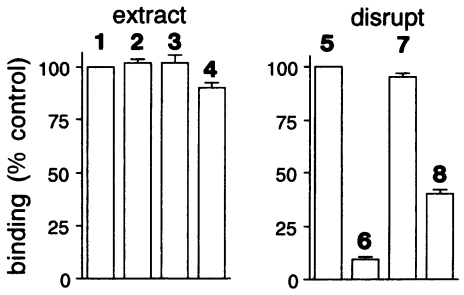

FIG. 4. Biochemical properties of the NLS binding site. Tobacco nuclei were allowed to associate with [^{14}C]O2WT either after (extract bars) or during (disrupt bars) incubation with labeling buffer (bars 1 and 5) or labeling buffer plus 0.25 M NaCl (bars 2 and 6), 1% Triton X-100 (bars 3 and 7), or 6 M urea (bars 4 and 8). Results are reported as percentage binding of control (±SE). Average total and nonspecific binding were 46,000 and 13,000 cpm, respectively.

effect on binding (22). Nuclei were treated for 15 min on ice with NaCl, Triton X-100, or urea prior to washing and NLS binding to determine whether the binding site could be extracted (Fig. 4, bars 1–4). The results confirm our earlier conclusion that the site is resistant to extraction and, thus, firmly associated with the nucleus (22). Nuclei were then treated before and during binding to determine whether the agents could disrupt association of NLS with the binding site (bars 5–8). The results showed that, compared to an untreated control, binding was inhibited 90% by NaCl and 60% by urea but was unaffected by Triton X-100.

As observed for binding, pretreatment of nuclei followed by DSS crosslinking demonstrated that the radiolabeled NBPs were also tightly associated with the nuclear pellet (compare Fig. 4, bars 1–4 with Fig. 5, lanes 1–4). Interestingly, pretreatment of the nuclei with urea reduced binding by 15% (Fig. 4, bar 4) compared to an untreated control, and this correlated with a reduction in labeling of the larger of the 50- to 60-kDa NBPs as well as an apparent mass change in at least one of the 30- to 40-kDa proteins (Fig. 5, lane 4). The reduction in labeling and mass was presumably caused by partial extraction of one of the NBPs or one or more unlabeled proteins that interact with the NBPs. As observed for binding, NBP labeling was sensitive to salt and urea but not to detergent (compare Fig. 4, bars 5–8 with Fig. 5, lanes 5–8). Overall, the tight correlation between the biochemical properties of the NLS binding site and the radiolabeled NBPs indicates that the NBPs constitute the binding site.

DISCUSSION

In a previous study of NLS binding to purified nuclei, we demonstrated that plants possess a low-affinity site that can

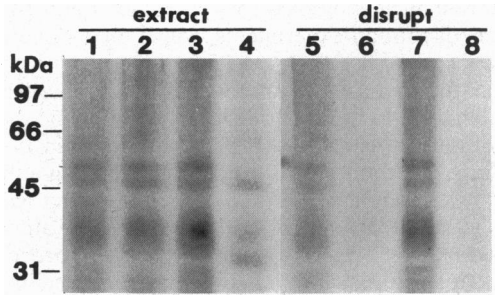

FIG. 5. Biochemical properties of tobacco nuclear NBPs. Fluorograph showing labeling with [^{14}C]O2WT and DSS either after (lanes 1–4) or during (lanes 5–8) incubation with labeling buffer (lanes 1 and 5) or labeling buffer plus 0.25 M NaCl (lanes 2 and 6), 1% Triton X-100 (lanes 3 and 7), or 6 M urea (lanes 4 and 8). For comparison, note that lanes are equivalent to bars shown in Fig. 4.

specifically bind to ^{14}C- and ^{125}I-labeled peptides to functional NLSs (22). Two classes of targeting signals, the SV40 large T-antigen NLS and the bipartite NLS from the plant transcription factor O2, were found to compete for this site. In addition, mutant sequences to these NLSs that do not function *in vivo* competed poorly for binding. Furthermore, we found that a SV40 large T-antigen NLS with the amino acids in reverse order, and presumably nonfunctional, competed poorly for binding compared to the native NLS. Because the reverse NLS retains the overall charge of the native signal, this indicates that basic charge is not the sole determinant of interaction with the NPC site (22). Based on its resistance to DNase I, salt, and detergent extraction, the site is a component of the lamina/pore fraction (24). In fact, immunolocalization of NLS binding by EM demonstrated that the site is at the NE and NPC (22). These findings strongly indicate the physiological significance of the NPC binding site.

For crosslinking, we have used radiolabeled O2WT because it displays only 10–25% nonspecific association in binding assays, whereas a SV40 large T-antigen NLS peptide displays a nonspecific binding component of 50% or greater of total binding (22). Thus, crosslinking with radiolabeled O2WT should result in reduced nonspecific labeling, in particular in comparison to the SV40 large T-antigen NLS, which has been used in numerous attempts to identify NLS binding factors in animals and yeast (reviewed in ref. 1). The polypeptides identified by crosslinking to O2WT constitute the NLS binding site by the following criteria: (*i*) the specificity of labeling with [^{14}C]- and [^{125}I]O2WT is essentially identical to that of the binding site, (*ii*) the apparent K_d for labeling based on the addition of competitors is similar to that of the binding site, (*iii*) the resistance of the labeled NBPs to extraction by salt and Triton X-100 indicate that they are also components of the lamina/pore fraction, and (*iv*) biochemical characteristics of the NBPs such as resistance to extraction and disruption of labeling are similar to the characteristics of the binding site. The relationship of the NBPs or the significance of several NBPs of two approximate size classes is unclear. It is possible, however, that some of the NBPs do not directly interact with NLSs but are in close proximity to these signals during the binding step of import.

Of the NBPs identified by chemical crosslinking, only the 54- and 56-kDa proteins identified by Adam *et al.* (25) have been demonstrated to have an involvement in nuclear import (26). One of the crosslinking reagents that we have used (i.e., DSS) is the same as that used by Adam *et al.*, and we detect two proteins of similar mass (50–60 kDa). However, our attempts to detect proteins of similar mass in tobacco or maize nuclei with antibodies to the NLS receptor of Adam *et al.* have not been successful (G.R.H., S. A. Adam, and N.V.R., unpublished data). Other NBPs have been identified in animals and yeast (reviewed in refs. 1 and 2; see also ref. 27) mostly by ligand blotting (28). This technique has been used often (1) but has yet to identify a protein that is a viable candidate for a NLS recognition component involved in nuclear import. The yeast protein NSR1 is a serine-rich phosphoprotein that is involved in ribosome biogenesis (29) and, in fact, possesses functional NLSs (30). The nucleolar protein Nopp140 is also serine-rich and phosphorylated and is reported to shuttle between the nucleus and cytoplasm (31), although its role in import is unclear. Interestingly, Rab17, a plant serine-rich phosphoprotein whose gene is expressed in response to the phytohormone abscisic acid also binds to NLSs via ligand blotting (32). Proteins identified as NBPs by other biochemical means include Hsc70 (33), which may have a role in nuclear import (34). We have excluded the possibility that the NLS binding site is Hsp70, Bip, or protein disulfide isomerase (22).

It is generally accepted from *in vitro* import systems that import in animals requires cytosolic factors (reviewed in ref. 1), although there is evidence to the contrary (35). It is also possible that the cytoskeleton may play a role in this process (reviewed in ref. 36). The NLS receptor of Adam *et al.* (26) is cytosolic but may be partially nuclear because a presumed homolog is found in NE fractions (25). Recently, a second soluble factor has been purified that, when incubated with the receptor, stimulates NLS binding to the NE in mammals (37). In yeast, there may not be a requirement for cytosol (38, 39), although a recent report contradicts this conclusion (40). In plants, this question is unanswered, and a recent report of a protease protection import assay in plants did not address this issue (8). Our binding and crosslinking data indicate that at least some component of NLS recognition occurs at the NPC in plants. Other differences between plants and animals may be possible, considering environmental extremes such as low temperatures at which many plants must survive and, presumably, sustain nuclear import. Binding at the NPC, however, does not exclude the possibility that soluble factors may stimulate NLS binding or that there are also soluble NBPs. We have examined several different cytosolic fractions from plant cells for NBPs by using both chemical and photoaffinity crosslinking reagents. No specific NBPs have yet been detected (unpublished data); however, factors that stimulate NPC binding may not have to associate directly with NLSs (37). Our study is an essential step in understanding the nuclear import apparatus of higher plants. Also, development of *in vitro* import systems in plants by an immunofluorescence approach should permit us to examine any requirement for cytosolic factors and functionally test purified plant NBPs.

The authors thank Kurt Stepnitz for photography. We also thank Antje Heese-Peck for critical reading of the manuscript and Stephane Lobreaux, Harley P. Smith, and Mark Shieh for helpful discussions. This work was supported by grants from the Department of Energy (DE-FG02-90ER20021) to N.V.R. and the National Science Foundation (BIR-9203692) to G.R.H.

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