Translocation of NLS–BSA conjugates into nuclei of permeabilized mammalian cells can be supported by protoplast extract

An experimental system for studying plant cytosolic factors involved in nuclear import

Yehoshua C. Broder[a], Ariel Stanhill[a], Nehama Zakai[a], Assaf Friedler[b], Chaim Gilon[b], Abraham Loyter[a,*]

[a] *Department of Biological Chemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel*
[b] *Department of Organic Chemistry, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel*

Received 11 June 1997

Abstract An heterologous experimental system, which allows the study of the yet unknown cytosolic factors involved in nuclear import of nuclear localization signal (NLS)-containing proteins in plants, has been established. The ability of plant cell extract to substitute mammalian cytosol and to promote translocation of NLS-containing proteins into nuclei of permeabilized HeLa cells was demonstrated. The results described in the present work show that nuclear import of fluorescently labeled BSA conjugates bearing the NLS sequence of SV40 large T antigen could be supported by petunia cell cytoplasmic extract. This heterologous system shows the characteristic features of the homologous mammalian system, namely, is ATP dependent and is inhibited by WGA, GTPγS as well as by non-fluorescent NLS–BSA conjugates. The system described here offers an experimental method to study and characterise cytosolic factors which are required for nuclear import in plants.

Key words: Nuclear import; Petunia extract; Permeabilized cell; Plant protoplast

1. Introduction

Soluble cytosolic proteins have been implicated in nuclear import of nuclear localization signal (NLS) bearing proteins in mammalian and in yeast cells [1,2]. This was mainly demonstrated by the use of permeabilized cells which are deprived of their cytosolic content but preserve the specific nuclear import machinery as well as the nuclear pore complex (NPC) structure [3].

Translocation of NLS-containing proteins into the nuclei of permeabilized cells is absolutely dependent on the addition of cytosolic extract, which contains several proteins that are required for the various steps of the transport process [3,4]. Most of these proteins have been isolated and characterised. Among them are: importin α,β which serves as an NLS receptor, NTF2, p97, and Ran/TC4, a small G-protein [reviewed in [1,2]]. In certain cells, heat shock proteins such as the hsp70 may also be involved in nuclear transport of NLS-containing proteins [5].

Proteins bearing NLS sequences, such as the maize regulatory protein, Opaque 2, as well as the maize transcriptional activator, R protein, have also been identified in plants [6–8]. The Vir E2 and Vir D proteins of *Agrobacterium tumefacience*, which probably mediate translocation of the bacterial T-DNA into nuclei of infected plant cells, also contain well-studied and characterised NLS sequences [[9] and reviewed in [10]]. However, very little is known regarding the existence and the specific function of cytosolic proteins of plant origin which may be involved in promoting specific nuclear import. This is mainly due to the absence of an appropriate experimental system, such as permeabilized plant cells, in which involvement of cytosolic factors can be followed and elucidated.

Recently, attempts have been made to prepare permeabilized plant protoplasts containing a functional intact nuclear import machinery [11] with the expectation that translocation of NLS-containing proteins into such nuclei will necessitate the addition of cytosolic extract, as was demonstrated with permeabilized yeast and mammalian cells [1,2]. Unfortunately, as was reported before [11], as well as described in the present paper, nuclear import of NLS-containing proteins into the nuclei of permeabilized protoplasts, although was relatively efficient, proceeded without any external additions. This was attributed to the presence of a residual cytosol, which apparently could not be removed from plant protoplasts during the permeabilization process [11].

In the present paper we suggest to use permeabilized mammalian cells as an alternative way to study and characterise cytosolic factors of plant origin. Our results clearly demonstrate that an extract obtained from petunia protoplasts can substitute a mammalian cytosol, such as reticulocyte lysate, in supporting nuclear entry of an NLS-containing protein into permeabilized HeLa cells. Translocation supported by petunia extract is ATP and temperature dependent and is inhibited by wheat germ agglutinin (WGA) as well as GTPγS.

2. Materials and methods

2.1. Materials

Sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexan-1-carboxylate (sulfo-SMCC) was purchased from Pierce Chemical Co., GTPγS, aprotinin, leupeptin, pepstatin and fluorescein isothiocyanate–dextran (FITC–dextran) from Sigma, lissamine rhodamine B sulfonylchloride from Molecular Probes and digitonin from Fluka.

*Corresponding author. Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. Fax: (972) 2-6586448.
E-mail: loyter@vms.huji.ac.il

PII S0014-5793(97)00855-7

2.2. Buffers

Lysis buffer contained 10 mM HEPES (pH 7.4), 10 mM potassium acetate, 2.5 mM EDTA, 3 mM DTT, 0.6 mM PMSF, and aprotinin/leupeptin/pepstatin (5 μg/ml of each).

Dialysis buffer contained 20 mM HEPES (pH 7.4), 110 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 2 mM DTT, 0.2 mM PMSF, and aprotinin/leupeptin/pepstatin (1 μg/ml of each).

Transport buffer (TB) contained 20 mM HEPES (pH 7.3), 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, and aprotinin/leupeptin/pepstatin (1 μg/ml of each).

CPW solution contained 10 mM $CaCl_2$, 0.2 mM KH_2PO_4, 2 mM $MgSO_4$, 1 mM KNO_3, 10% mannitol (w/v), pH 5.7 [12].

2.3. Cell culture

HeLa cells were grown as monolayers in DMEM growth medium supplemented with 10% fetal calf serum and with 100 U/ml penicillin and 0.1 mg/ml streptomycin (Biological Industries, Beit-Haemek, Israel). The cells were maintained in a humidified incubator with 5% CO_2 atmosphere and were removed by trypsinization and replated on glass coverslips 24–48 h before use [13].

Protoplasts were prepared from 3-day-old petunia hybrida cultured cells (line p-3704) as described before [14].

2.4. Synthesis of NLS peptides

A synthetic peptide containing the NLS sequence of the SV40 large T antigen: PKKKRKVC, [15] was synthesized by SPPS on Rink Amide resin (loading 0.5 mmol/g), using Applied Biosystems Peptide Synthesizer model 433A. The peptide was purified by HPLC and characterised by TOF-MS [16].

2.5. Preparation of fluorescently labeled NLS–bovine serum albumin (FL–NLS–BSA) conjugates (transport substrate)

BSA (60 mg) was dissolved in 3 ml of 100 mM NaCl and 50 mM $NaHCO_3$, pH 9.0, to which 1.5 mg lissamine rhodamine B sulfonyl chloride was added. The mixture was stirred for 3 h at room temperature and the unincorporated fluorochrome was removed from the FL-BSA by gel filtration (Sephadex G-25, prewashed with PBS, pH 7.4). The synthetic peptide bearing the NLS of the SV40 large T antigen was covalently linked to the lissamine rhodamine BSA molecule via the C-terminal cysteine residue, using the heterobifunctional cross-linker sulfo-SMCC. Each milligram of lissamine rhodamine–BSA was incubated with 0.15 mg of sulfo-SMCC for 90 min at room temperature. Unlinked sulfo-SMCC was removed by Sephadex G-25 column and the conjugate was mixed with the synthetic NLS–peptide in a 2:1 (w/w) ratio and incubated over night at 4°C. At the end of the incubation period, cysteine (5 mM) was added for 1 h at room temperature and the unincorporated peptide was removed by gel filtration on Sephadex G-25 column.

2.6. Preparation of a cytosolic extract from petunia protoplasts

Freshly prepared petunia protoplasts (from 400 ml of 3-day-old petunia cell suspension) were centrifuged (5 min, 300×*g*) at 4°C, washed twice with cold CPW [12] and the packed pellet obtained (9 ml) was resuspended in an equal volume of cold Lysis buffer and then incubated for 10 min at 4°C to allow cell swelling. The swollen cells were homogenized by six strokes in a 40 ml glass homogenizer (Kontes Glass Co., Vineland, NJ) until at least 70% of the cells appeared broken by light microscopy. The resulting homogenate was centrifuged for 15 min (1500×*g*) at 4°C and the supernatant obtained was centrifuged again (100 000×*g*, 35 min, 4°C in a Beckman Optima TLX ultracentrifuge). To reach a concentration of 20–40 mg protein/ml, the clear solution obtained was concentrated and dialyzed against a dialysis buffer using a collodion membrane apparatus (molecular mass cutoff 10 000 Da; Schleicher and Schuell, Inc., Keene, NH). Following several changes of the dialysis buffer, samples were frozen in liquid nitrogen and stored at −80°C. Protein concentration was determined according to Bradford [17].

2.7. Permeabilization of petunia hybrida protoplasts and incubation with FL–NLS–BSA molecules

Two alternative methods have been used to obtain permeabilized protoplasts. Petunia protoplasts prepared as before were washed twice in CPW, then were dialyzed for 3 h against an hypotonic medium, namely, CPW in which the mannitol concentration was decreased from 10% to 2%. Alternatively, petunia protoplasts were washed once in CPW from which $CaCl_2$ was omitted and then suspended in the same buffer to a final concentration of 50% (v/v) and incubated for 2 h at room temperature. About 80% of the protoplasts in both suspensions appeared permeabilized, namely allowed penetration of fluorescently labeled high and low molecular weight dextrans (150, 9 kDa respectively) to the cytoplasm but only the low molecular weight dextran penetrated the intranuclear space (not shown) [18]. Based on these observations, we concluded that such petunia protoplasts are permeabilized while their nuclei remain intact. In this respect our permeabilized protoplasts resemble digitonin treated mammalian cells [13]. For estimation of nuclear import, a volume of 25 μl of permeabilized protoplasts suspension (50% v/v in CPW solution without $CaCl_2$, supplemented with an energy regeneration system containing 0.2 mM GTP, 1.5 mM ATP, 9 mM creatine phosphate, 1.2 U creatine phosphokinase) were incubated at room temperature for 20 min with 10 μg of FL–NLS–BSA (complete system). Following fixation with 3.8% (w/v) formaldehyde, chromosomal DNA was fluorescently stained by 4′,6-diamidino-2-phenylindole (DAPI) [19] and the cells were observed by Leitz Wetzlar fluorescent microscope.

2.8. Permeabilization of cultured HeLa cells and nuclear import of FL–NLS–BSA conjugates

Cultured HeLa cells were grown on 10 mm coverslips to a subconfluent density and then were permeabilized with digitonin (33 μg/ml, final concentration) essentially as previously described [13]. Nuclear import of the FL–NLS–BSA conjugates into the nuclei of the permeabilized HeLa cells was followed by a fluorescent microscope as described [13]. Briefly, the coverslips containing the permeabilized cells were placed on top of a drop (50 μl) which contained a transport buffer (TB), energy regenerating system (0.2 mM GTP, 1.5 mM ATP, 9 mM creatine phosphate, 1.2 U creatine phosphokinase) and 2–4 μg of FL–NLS–BSA conjugates. Following 15 min incubation in a humidified chamber at 30°C, the coverslips were transferred to a second drop which contained 20 μl of either rabbit reticulocyte lysate (Promega, USA) or petunia cells extract, (prepared as described above in Section 2.6) to which 20 μl of the first drop was added, to give a final volume of 40 μl (complete system). At the end of 60 min incubation in the humidified chamber, nuclear import was terminated by washing the coverslips with TB and then the permeabilized cells were fixed with 3.8% (w/v) formaldehyde. Chromosomal DNA was fluorescently stained by DAPI [19] and the fixed cells were observed by Leitz Wetzlar fluorescent microscope.

3. Results and discussion

3.1. Nuclear import in permeabilized petunia protoplasts does not require cytosolic factors

The use of permeabilized mammalian cells was instrumental in the discovery and the characterization of animal and yeasts cytosolic factors which mediate nuclear import of proteins containing NLS sequences [1,2]. Also in plants, proteins containing NLS sequences might interact with putative, yet uncharacterised, cytosolic factors and thus form an active translocation complex. Indeed, recently extensive efforts have been made in our laboratory as well as in others [11] to obtain permeabilized protoplasts with intact nuclei, in order to establish an experimental system for studying the role of soluble cytosolic factors in the nuclear import machinery of plants. Essentially, two methods (with various modifications) have been used in our laboratory for the preparation of permeabilized petunia protoplasts (see Section 2). Protoplasts were either incubated in a medium lacking calcium ions or alternatively were osmotically swollen in hypotonic medium. Both methods resulted in permeabilized protoplasts which allowed the entry of high molecular weight molecules (fluorescently labeled dextran of 150 kDa) into the extra- but not to the intranuclear space [18], indicating that the nuclear envelope was intact in our experiments. We have used a synthetic pep-

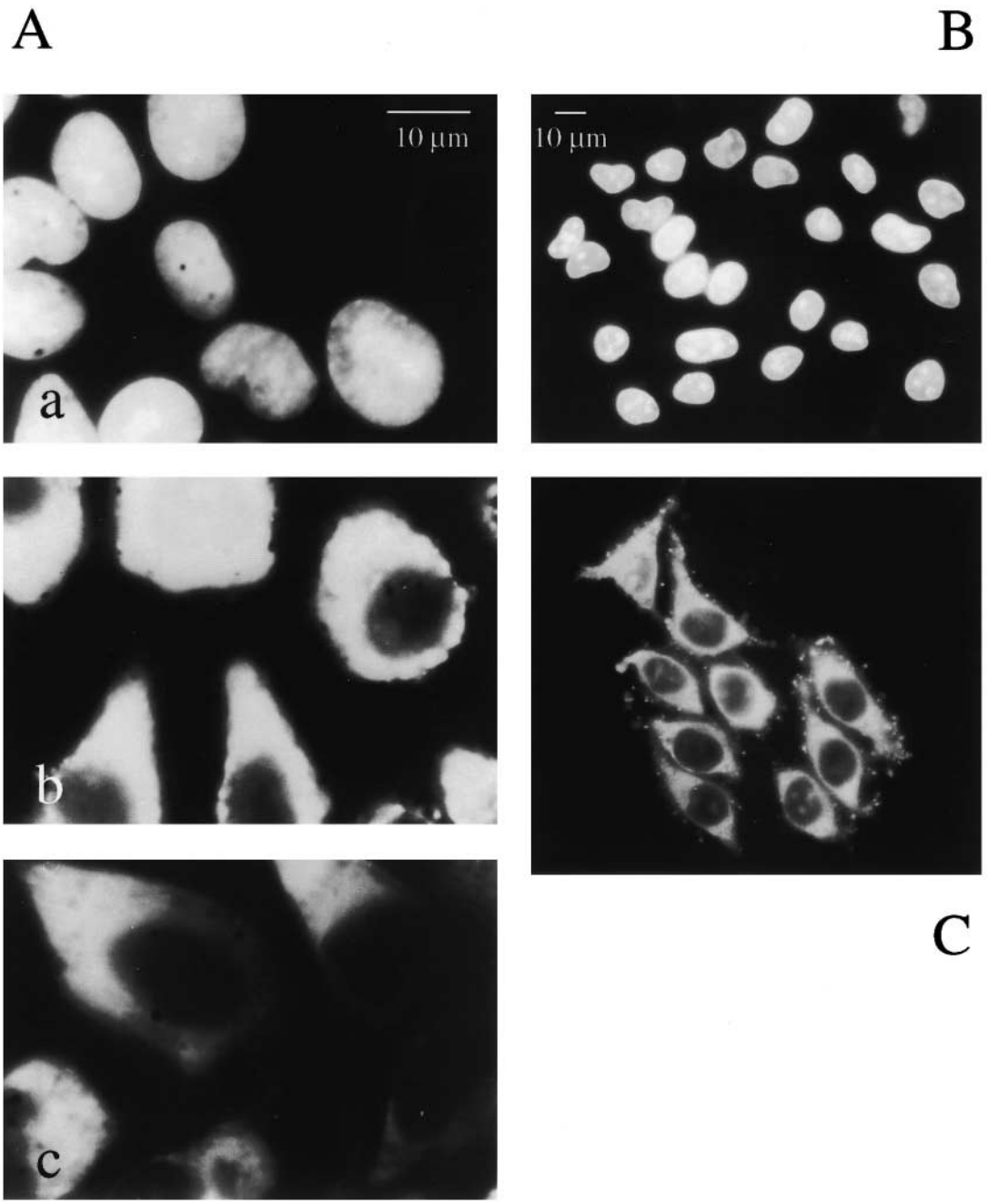

Fig. 1. Translocation of FL–NLS–BSA conjugates into nuclei of permeabilized HeLa cells in the presence of petunia cytosolic extract: fluorescent microscopic observations. Permeabilized HeLa cells were incubated with FL–NLS BSA as described in Section 2 in the presence of (A) petunia cell extract (B) reticulocyte extract (C) in the absence of cytosolic extract. (Aa) complete system. (Ab) ATP depleted cells. (Ac) as in (Aa) but at 4°C. All other experimental conditions were as described in Table 1 and 2.

tide bearing the NLS sequence of the SV40 large T antigen, since previous experiments clearly demonstrated that this sequence is also active in plant cells, namely, promotes translocation of proteins bearing the NLS of SV40 large T antigen into plant nuclei [20,21].

The results summarized in Table 1 show that FL–NLS–BSA molecules accumulated within the protoplast nuclei while those lacking the NLS synthetic peptide failed to do so, giving the impression that the observed nuclear import is specific and NLS dependent. However, nuclear import in petunia protoplasts did not require any external additions such as cytosolic extract: about the same fluorescent intensity appeared in the petunia nuclei in the absence or presence of cytosolic extract (Table 1). Furthermore, nuclear import was observed in ATP-depleted protoplasts and was not inhibited by the lectin WGA (Table 1). These observations are in contrast to translocation of NLS-containing proteins into nuclei of permeabilized mammalian cells, which is absolutely dependent on the presence of external cytosolic factors, requires metabolic energy and is inhibited by WGA (ref. [3] and Table 1). Very similar results to those obtained in the present work have recently been published using permeabilized Tobacco protoplasts [11]. To explain such results the authors suggested that a certain amount of the cytosolic factors which are needed for

Table 1
Accumulation of FL–NLS–BSA in nuclei of permeabilized petunia protoplasts and in permeabilized HeLa cells: comparative studies

Experimental conditions		Accumulation in nuclei	
		Petunia protoplasts	Cultured HeLa cells
A	Complete system+FL–NLS–BSA	+	+
B	Complete system+FL–BSA	−	−
C	As in (A) without the addition of reticulocyte lysate	+	−
D	As in (A) in the presence of Hexokinase+glucose (ATP depletion)	+	−
E	As in (A) in the presence of WGA	+	−

Experimental conditions of permeabilization of petunia protoplasts and of cultured HeLa cells, nuclear import of FL–NLS–BSA conjugates (complete system: see Section 2, Section 2.6 for petunia protoplasts and Section 2.7 for HeLa cells) as well and fluorescent microscope observations were as described in Section 2.
+, At least 50% of the nuclei are fluorescently labeled.
−, Less then 5% of the nuclei are fluorescently labeled.
For ATP depletion 1.5 U/μl hexokinase and 10 mM glucose were used.

the translocation process, did not leak during the permeabilization process and exert their effect within the permeabilized protoplasts. If true, this may explain the present and the previous observations [11] regarding the accumulation of NLS-containing proteins within the protoplasts nuclei in the absence of any external cytosolic factors. It is also possible, as was suggested [11] that nuclear import in plant cells is characterised by unique features and neither requires ATP nor is inhibited by WGA. The alternative possibility, that nuclei in such protoplasts are partially leaky and allow unspecific accumulation of FL–NLS–BSA molecules, cannot be excluded. Due to the positively charged amino acids present within the NLS sequence, the NLS–BSA conjugates may unspecifically interact with the negatively charged chromosomal DNA and consequently be retained within the intranuclear space. Evidently, such process will be ATP and cytosolic factors independent but NLS dependent. It is clear, however, that permeabilized protoplasts possessing such features cannot serve as an experimental system for studying the existence and characteristics of cytosolic proteins which may be involved in nuclear import in plant cells.

3.2. Petunia extract can replace reticulocyte cytosol in promoting nuclear import of NLS-containing proteins in permeabilized HeLa cells

As evident from the fluorescent micrographs depicted in Fig. 1, an extract obtained from Petunia protoplasts supported translocation of the FL–NLS–BSA conjugates into nuclei of permeabilized HeLa cells. Identical results were also obtained with permeabilized cells prepared from a suspension of human colon cells (not shown). These results (Tables 1 and 2) show that nuclear import in the present heterologous system exhibits the same features that characterise translocation of NLS-containing proteins in the homologous system, namely in the presence of mammalian cytosolic extract [3]. As can be seen, nuclear import promoted by the plant extract was NLS dependent, required metabolic energy and was inhibited by the lectin WGA (Table 2). This is inferred from our microscopic observations, showing a very little intranuclear fluorescence in ATP depleted permeabilized cells nuclei or in those incubated in the cold. As expected, most of the fluorescence in these systems was retained in the extranuclear space (Fig. 1). Translocation of the FL–NLS–BSA in the present heterologous system was competitively inhibited by non-fluorescently labeled BSA molecules bearing the NLS of the SV40 large T antigen but not by BSA molecule lacking an NLS sequence. These observations clearly indicate the involvement of specific soluble NLS receptors in the nuclear import machinery of plants.

4. Conclusions

Our present results clearly show that nuclear import of NLS-containing proteins can be achieved in an heterologous system, namely in a combination of permeabilized mammalian (HeLa) cells and plant cell extract. It is our view that the present heterologous system, features of which are very similar to those of the mammalian homologues system [3], offers a convenient and practical method to study, characterise and follow the biological function of plant cytosolic factors which intracellularly participate in nuclear import of NLS containing proteins. The existence of such receptors in plants have

Table 2
Petunia cell extract resembles reticulocyte lysate in its ability to support nuclear import in permeabilized HeLa cells

Experimental conditions	Translocation of FL–NLS–BSA in the presence of Cytosol extracted from	
	Petunia protoplasts	Reticulocytes
4°C	−	−
30°C	+	+
In the presence of		
GTPγS	−	−
Hexokinase+glucose (ATP depletion)	−	−
NLS–BSA	−	−

Permeabilization of HeLa cells and nuclear import assay were conducted as described in Sections 2 and 2.7.
For GTPγS treatment, 500 μM and 100 μM GTPγS were added to the petunia cytosol and to the reticulocyte lysate, respectively, before incubation with the cells. ATP depletion was as in Table 1. For the competition experiments the ratio of Fl–NLS–BSA: NLS–BSA (not fluorescent) was 1:3 (w/w).
+ and −, Presence or absence of fluorescently labeled nuclei, respectively, as described in Table 1.

been suggested before [11]. This can also be concluded from our experiments showing that NLS peptides compete with FL–NLS–BSA on translocation into HeLa cells nuclei. G-proteins may also be involved in the plant nuclear import machinery. However, as much as 500 μM of GTPγS were required to inhibit nuclear import in the heterologous system as compared to 100 μM in the homologues one (Table 2 and ref. [22]). This may indicate that the G-proteins which are involved in translocation of NLS-containing proteins in plant cells, may be different in their structure and characteristics from those isolated from animal cells [1,2]. Current experiments are conducted in our laboratory, using the present described system, to isolate the various plant cytosolic factors present in the petunia extract as well as the putative G-proteins and to study their specific features.

As mentioned above, previous results have clearly shown that the NLS sequence of SV40 large T antigen is recognized by the plant nuclear import machinery [20,21]. In addition, microinjection of Mcm 1 protein, a nuclear protein from *S. Cerevisia*, into *Xenopus laevis* frog oocytes resulted in its nuclear import, demonstrating that an NLS of Yeast protein can be recognized by the nuclear transport machinery of a higher eukaryotic cell [23]. Our present results demonstrate a new aspect of the nuclear import mechanism. A plant cytosol is able to support translocation of an NLS protein into mammalian cell nuclei, demonstrating probably that mammalian NPC as well as their cytoskeleton network are able to recognize plant cytosolic factors. These results, as well as the above mentioned observations [23], strongly support the concept that the mechanism by which proteins are translocated into nuclei is functionally conserved among all eukaryotes. It will certainly be of interest to study and to compare the molecular structure as well as the detailed activity of the putative plant cytosolic factors, which can be characterised by the present described system, to those that have already been isolated from mammalian cells.

Acknowledgements: This work was supported by a grant from BSF No. 93-00028.

References

[1] Powers, M.A. and Forbes, D.J. (1994) Cell 79, 931–934.
[2] Melchior, F. and Gerace, L. (1995) Curr. Opin. Cell Biol. 7, 310–318.
[3] Adam, S.A., Sterne-Marr, R. and Gerace, L. (1990) J. Cell Biol. 111, 807–816.
[4] Görlich, D., Prehn, S., Laskey, R.A. and Hartmann, E. (1994) Cell 79, 767–778.
[5] Shi, Y. and Thomas, J.O. (1992) Mol. Cell Biol. 12, 2186–2192.
[6] Hicks, G.R. and Raikhel, N.V. (1995) Ann. Rev. Cell Dev. Biol. 11, 155–188.
[7] Varagona, M.J., Schmidt, R.J. and Raikhel, N.V. (1992) Plant Cell 4, 1213–1227.
[8] Sheih, M., Wessler, S.R. and Raikhel, N.V. (1993) Plant Physiol. 101, 353–361.
[9] Citovsky, V., Zupan, J., Warnick, D. and Zambryski, P. (1992) Science 256, 1802–1805.
[10] Citovsky, V. and Zambryski, P. (1993) Annu Rev Microbiol 47, 167–197.
[11] Hicks, G.R., Smith, H.M.S., Lobreaux, S. and Raikhel, N.V. (1996) Plant Cell 8, 1337–1352.
[12] Frearson, E.M., Power, J.B. and Cocking, E.C. (1973) Dev. Biol. 33, 130–137.
[13] Adam, S.A., Sterne-Marr, R. and Gerace, L. (1992) Methods Enzymol. 219, 97–110.
[14] Zakai, N., Ballas, N., Hershkovitz, M., Broido, S., Ram, R. and Loyter, A. (1993) Plant Mol. Biol. 21, 823–834.
[15] Kalderon, D., Richardson, W.D., Markham, A.F. and Smith, A.E. (1984) Nature (London) 311, 33–38.
[16] McIver Jr., R.T., Li, Y. and Hunter, R.L. (1994) Proc. Natl. Acad. Sci. USA 91, 4801–4805.
[17] Bradford, M.M. (1976) Anal. Biochem. 116, 53–54.
[18] Duverger, E., Pellerin-Mendes, C., Mayer, R., Roche, A.C. and Monsigny, M. (1995) J. Cell Sci. 108, 1325–1332.
[19] Williamson, D.H. and Funnell, D.J. (1975) Methods Cell Biol. 12, 335–351.
[20] Lassner, M.W. (1991) Plant Mol. Biol. 17, 229–234.
[21] van der Krol, A.R. and Chua, N.H. (1991) Plant Cell 3, 667–675.
[22] Melchior, F., Paschal, B., Evans, J. and Gerace, L. (1993) J. Cell Biol. 123, 1649–1659.
[23] Wagner, P. and Hall, M.N. (1993) FEBS Lett. 321, 261–266.