

Nuclear import of DNA in digitonin-permeabilized cells

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SUMMARY

DNA can enter intact mammalian nuclei with varying degrees of efficiency in both transfected and microinjected cells, yet very little is known about the mechanism by which it crosses the nuclear membrane. Nucleocytoplasmic transport of fluorescently labeled DNA was studied using a digitonin-permeabilized cell system. DNA accumulated in the nucleus with a punctate staining pattern in over 80%

of the permeabilized HeLa cells. Nuclear localization of the labeled DNA was energy dependent and occurred through the nuclear pore, but did not require the addition of soluble cytoplasmic protein factors necessary for protein import.

Key words: Nuclear transport, Fluorescent DNA, Digitonin permeabilized cell

INTRODUCTION

Nucleocytoplasmic trafficking of cellular molecules occurs by both passive diffusion and signal-mediated processes through the nuclear pore complex, yet for all large intracellular macromolecules (>40-60 kDa) an energy and signal-dependent mechanism is required. Both nuclear localizing proteins and some RNAs, in the form of small nuclear ribonucleoprotein complexes (snRNPs), require a specific amino acid sequence (nuclear localizing signal, NLS) to cross the nuclear envelope via the nuclear pore complex (Gorlich and Mattaj, 1996). While the mechanisms that each of these use are distinct, both require energy and soluble cytoplasmic proteins to facilitate nuclear entry (Fischer et al., 1993; Marshallsay and Luhrmann, 1994; Michaud and Goldfarb, 1992).

Nuclear import of NLS-bearing proteins is a multi-step process mediated by several cytoplasmic factors (Gorlich and Mattaj, 1996). First, the nucleophilic protein binds the karyopherin α/β heterodimer in the cytoplasm. This complex then docks to the cytoplasmic face of the nuclear pore via the β subunit (Gerace, 1992). The GTPase Ran mediates the subsequent energy-dependent translocation of the docked transport substrate through the pore complex (Koepp and Silver, 1996).

The various snRNPs, which have intron splicing functions in mRNA processing, also enter and exit the nucleus under the influence of signaling mechanisms. hnRNP A1, which contains the M9 transport signal, is imported using a karyopherin-independent pathway mediated by transportin (Pollard et al., 1996). Most snRNP import is mediated by interactions of the snRNA m³G cap structure with cytosolic proteins containing nuclear localization signals, and/or binding of Sm proteins to the Sm binding region of the snRNA (Fischer and Luhrmann, 1990; Marshallsay and Luhrmann, 1994; Michaud and Goldfarb, 1992). Export seems to be mediated by signals on the same RNPs, which have been observed to shuttle back

and forth between the nucleus and cytoplasm (Katz et al., 1994; Michael et al., 1995).

Digitonin-permeabilized cells have been a powerful system for studying the nuclear import of karyophilic proteins (Adam et al., 1991) and snRNA (Marshallsay and Luhrmann, 1994) in mammalian cells. The present study followed directly the nuclear transport of fluorescently labeled DNA in digitonin-permeabilized cells. DNA nuclear entry was energy dependent and occurred via the nuclear pore complex, but contrary to the mechanism of nuclear transport for other macromolecules, it did not require the addition of soluble cytoplasmic factors.

MATERIALS AND METHODS

PCR labeling of DNA

Texas Red-labeled dCTP (TR-dCTP) was incorporated into DNA using the polymerase chain reaction (PCR). The reaction mixture consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 5 μ M Texas Red-labeled dCTP (DuPont NEN, Boston, MA), 20 μ M dCTP, 25 μ M dATP, 25 μ M dGTP, 25 μ M dTTP (Pharmacia, Piscataway, NJ), 0.8 μ M of each primer, 0.2 pM template and 2.5 units of AmpliTaq (Perkin Elmer, Norwalk, Connecticut) or Ex Taq (PanVera, Madison, WI) polymerase per 100 μ l of PCR reaction. Cycling parameters were calculated based on template length and annealing temperature of the primers. Purification of amplified labeled DNA consisted of a chloroform extraction when mineral oil was present, and in all cases by washing on a Millipore Ultrafree-MC filter (100 kDa cut-off) in 'base import buffer' (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA). Samples were washed a minimum of 10,000-fold, and selected filtrates were checked for the presence of fluorescence using both agarose gel electrophoresis and spectrofluorometry. The number of Texas Red molecules present on each DNA molecule was determined to be 5-20 per 1,000 base pairs by quantitative spectrofluorometry, using TR-dCTP as a standard. Fluorescein-DNA (F-DNA), Cy5-DNA and biotin-DNA were all generated using PCR under the same conditions as TR-DNA synthesis, with the replace-

ment of TR-dCTP by fluorescein-dCTP (DuPont NEN), Cy5-dCTP (Amersham, Arlington Heights, IL) and biotin-dCTP (Gibco-BRL, Gaithersburg, MD), respectively. 932 bp, 2,015 bp, 2,999 bp and 5,066 bp TR-DNAs were generated using a common 3' primer (5' GGT GCT GAA AGC GAG GCT TTT TGG CCT CTG T 3') specific for lambda phage DNA. The 5' primers varied for the different fragments: 932 bp, 5' TAA AGT TCT CGG CAT CAC CAT CCG TCG GCA 3'; 2,015 bp, 5' CAT ACA CAA TGG TCG GGT CAA TCC CGC CAG T 3'; 2,999 bp, 5' GCG CAA GCT TGG GCG ATG ACT GAG CCG GA 3'; 5,066 bp, 5' GAC AGG CCG GTA TAT GCC GAC ACC TTC T 3'.

Fluorescent oligonucleotides and proteins

The 37-base oligonucleotide was synthesized with a 5' amino C6 modifier (Integrated DNA Technologies, Coralville, IA) and subsequently labeled with tetramethylrhodamine isothiocyanate (Sigma, St Louis, MO) and purified by column chromatography (Sephadex G25, Pharmacia). Fluorescein-dextran and fluorescein-dextran sulfate were purchased from Molecular Probes (Eugene, OR). NLS-allophycocyanin and NLS-streptavidin were created by crosslinking a synthetic peptide containing the SV40 large T antigen nuclear localizing sequence (CGYGPKKKRKVG) (Lanford et al., 1986) in a 20-fold molar excess to allophycocyanin (Calbiochem, La Jolla, CA) and streptavidin (Sigma), respectively, using the heterobifunctional crosslinker sulfo-SMCC (Pierce, Rockford, IL). Conjugation was carried out as described by Adam (Adam et al., 1991). Allophycocyanin has a natural fluorescence (Absorbance λ_{\max} = 650 nm, Emission λ_{\max} = 660 nm). The streptavidin was fluorescently labeled by mixing it with 100-fold molar excess of fluorescein-biotin (Sigma) and removing the excess fluorescein-biotin using Millipore Ultrafree-MC filters (30 kDa cut-off).

Nuclear import assays

Transport assays were performed as previously described (Adam et al., 1991) with minor modifications. HeLa cells were plated onto non-coated glass coverslips the day before so that they were 60-70% confluent for the assay. The cells were permeabilized using 40 μ g/ml digitonin in import buffer (Boehringer-Mannheim, Indianapolis, IN) and washed in import buffer, which consisted of degassed base buffer (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA) with the addition of 2 mM DTT and 1 μ g/ml each of aprotinin, pepstatin A and leupeptin (Sigma). Coverslips were inverted on a 50 μ l drop of complete import mixture on a sheet of Parafilm in a humidified plastic box. The complete import mixture contained import buffer with 0.5 mM ATP, 0.2 mM GTP, 5 mM creatine phosphate, 1 unit creatine phosphokinase (Calbiochem), transport substrate and either 50% rabbit reticulocyte lysate (RRL) (Promega Corp. Madison, WI) or 10 mg/ml BSA (Sigma). After incubation at either 37°C or 4°C, an additional 100 μ l import buffer was carefully pipetted under the coverslip to aid in removal of the coverslip from the Parafilm. After washing with import buffer, cells were fixed with 2% formaldehyde for 10-30 minutes and mounted on slides in import buffer containing 0.1% *p*-phenylenediamine dihydrochloride (Sigma). The nuclei and their nucleolar regions were identified by staining with 1 μ g/ml Hoechst 33258 (Sigma) in import buffer for 10 minutes. The coverslips were sealed to the slide with clear nail polish. Nail polish was also used to create a raised border on the slide to prevent shearing of the cells. Images of the samples were collected the same day by confocal microscopy (Bio-Rad MRC600 (krypton/argon laser) on a Nikon Optiphot, $\times 60$ oil plan apo objective with NA 1.4), or by epifluorescence microscopy on a Nikon Optiphot with a SenSys CCD Camera (Photometrics, Tuscon, AZ), $\times 60$ oil plan apo objective with NA 1.4.

To analyze the DNA remaining in the complete import buffer after the assay, the 150 μ l (50 μ l of original complete import mixture and 100 μ l of import buffer added after the assay) was removed from the

Parafilm with a pipetman. It was then extracted with phenol and then chloroform/isoamyl alcohol (24:1, v:v). After ethanol precipitation, the pellet was washed with 70% ethanol, dried, and dissolved in 10 μ l of water. The whole sample was analyzed on a 1% agarose gel.

HeLa cells were microinjected in the cytoplasm as previously described using an IM 200 microinjector (Narishige, Tokyo) (Dowty et al., 1995). The microinjection volume was sufficient to swell the cell. The microinjected cells were fixed in 2% formaldehyde and mounted in PBS containing 40% glycerol and 0.1% *p*-phenylenediamine.

Inhibitors of nuclear transport

Some of the digitonin-permeabilized cells were exposed for 10 minutes to 1 mg/ml WGA (United States Biochemical, Cleveland, OH) in import buffer containing 10 mg/ml BSA prior to performing the assay. In addition, 1 mg/ml WGA was added to the complete import mixture. *N*-ethylmaleimide (NEM) treatment of the cells consisted of incubating the digitonin-permeabilized cells for 10 minutes in 5 mM NEM (Sigma) followed by quenching for 5 minutes in 10 mM DTT. Mock-treated samples were incubated in 10 mM DTT for 15 minutes. The import reactions were depleted of energy by adding 1 unit of Apyrase (Sigma) and omitting the ATP, GTP, creatine phosphate and creatine phosphokinase. EDTA exposure was performed by adding it to a final concentration of 5 mM in the complete import mixture.

The effect of RNA on DNA transport was assessed by adding 10 μ g total pig spleen RNA to the complete import mixture. The RNA was prepared using RNazol solution (Cinna/Biotech Laboratories, Inc, Houston, TX). Dextran sulfate effects were determined by adding to the complete import mixture 10 μ g of dextran sulfate (Sigma) that was either 5 kDa, 10 kDa or 500 kDa in size.

The quantitative effect of the inhibitory conditions on TR-DNA and protein nuclear import was assessed by determining the number of fluorescent spots and the mean pixel value of undamaged nuclei, respectively. The analysis was done using a Power Macintosh 9500/120 computer and the public domain NIH Image program (developed at the US National Institutes of Health and available from the Internet at <http://rsb.info.nih.gov/nih-image/>). Differences among treatment groups were assessed by one-way ANOVA. When *F* tests from ANOVA indicated significant differences ($P < 0.05$), multiple comparisons were performed to compare group means using the least squares method. All of the analyses were done by the GLM (General Linear Models) procedure in SAS.

Nuclei isolation and Southern blot analysis

14 transport assays were performed with 4 μ g/ml PCR DNA without RRL extract with 45-minute incubations. After the assay, the cells were washed as above, but instead of fixing, nuclei were isolated according to the procedure in *Current Protocols* (Ausubel et al., 1993), with minor modification. The cells were scraped from the 14 coverslips into 1 ml import buffer each and combined. The combined cells were then centrifuged at 500 *g* for 5 minutes at 4°C. The supernatant was removed and 1.5 ml NP-40 Lysis Buffer A (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% v/v NP-40) was added. The lysed cells were then incubated on ice for 5 minutes, pulled through a 27-gauge needle, and centrifuged at 500 *g* for 5 minutes at 4°C. The nuclear pellet was resuspended in 1 ml NP-40 Lysis Buffer A and spun at 500 *g* for 5 minutes at 4°C. DNA was isolated from the nuclei as described for mammalian tissue (Ausubel et al., 1993). 2.5 μ g of the purified genomic/PCR DNA (quantitated spectroscopically) was digested with *EcoRI* (which does not digest the PCR DNA), and loaded into the well of a 1% agarose gel. After ethidium bromide staining of the gel to verify DNA digestion, a Southern blot was performed as described (Ausubel et al., 1993) using a vacuum blotter (LKB: Midton Acrylics Limited; Lockgilthead, Scotland). The 932 bp DNA was used to generate a ³²P-labeled probe using the Random Prime-It Kit (Stratagene, La Jolla, CA).

RESULTS

DNA accumulates in the nucleus of digitonin-permeabilized HeLa cells

Digitonin-permeabilized HeLa cells were exposed for varying amounts of time (0-60 minutes) at 37°C to a linear 932 bp, double-stranded DNA molecule (molecular mass approx. 621 kDa). Confocal fluorescence microscopic analysis was performed on the permeabilized cells incubated with TR-DNA in 'import buffer' without any cellular or cytoplasmic extract. Following a 60-minute incubation with 4 µg/ml of TR-DNA, about 80% of the nuclei exhibited a distinctive non-nucleolar punctate staining pattern with a small amount of diffuse nuclear staining (Fig. 1A). Labeled DNA entered the nucleus within 15 minutes and by 45 minutes the amount of TR-DNA in the nuclei reached the maximum level. The nuclei were deemed intact since fluorescein-dextran (molecular mass approx. 500 kDa), allophycocyanin (molecular mass approx. 105 kDa), NLS-allophycocyanin or NLS-streptavidin (molecular mass approx. 70 kDa) all failed to access the nucleoplasm under similar conditions (i.e. import buffer without extract) (data not shown). Much of the TR-DNA signal remained in the cytoplasm, consistent with the cytoplasmic sequestration previously noted for labeled DNA microinjected into myotubes in culture (Dowty et al., 1995). A similar nuclear staining pattern was observed with 932 bp DNA labeled with Cy5, fluorescein or biotin (data not shown), which indicated

that the staining pattern was not a consequence of the Texas Red molecule. Identical staining patterns were observed when nuclei were exposed to the 932 bp fragment at lower concentrations (20-200 ng/ml) (data not shown). Fluorescently labeled DNAs with different sequences were synthesized and were able to enter the nuclei with similar efficiencies, which indicated that this nuclear transport is not sequence-dependent (data not shown). DNA nuclear uptake was also observed in digitonin-permeabilized 3T3, COS and C2C12 cells (data not shown).

Fluorescein-dextran sulfate (molecular mass approx. 500 kDa) was used to determine if a similarly sized polyanion could

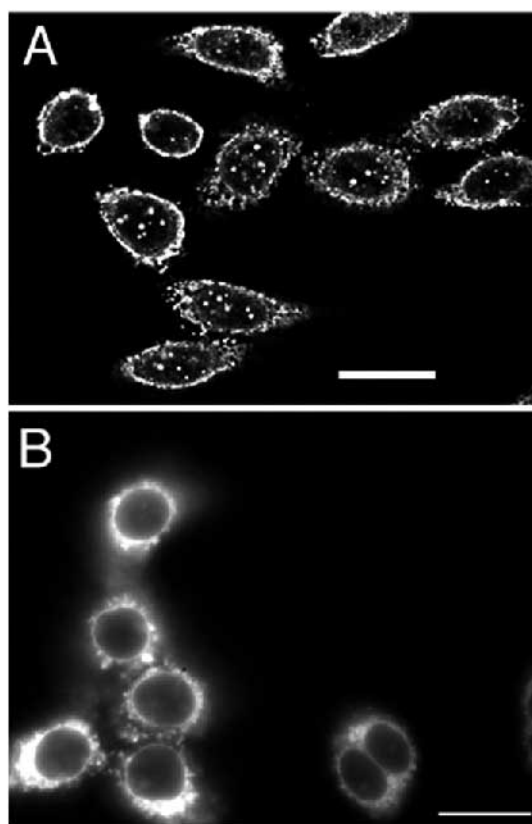


Fig. 1. Digitonin-permeabilized HeLa cells were exposed without rabbit reticulocyte lysate (RRL) to either 4 µg/ml of 932 bp TR-DNA (A) or 20 µg/ml fluorescein dextran sulfate (500 kDa) (B). A is a confocal fluorescence and B is an epifluorescence image. Bars, 25 µm.

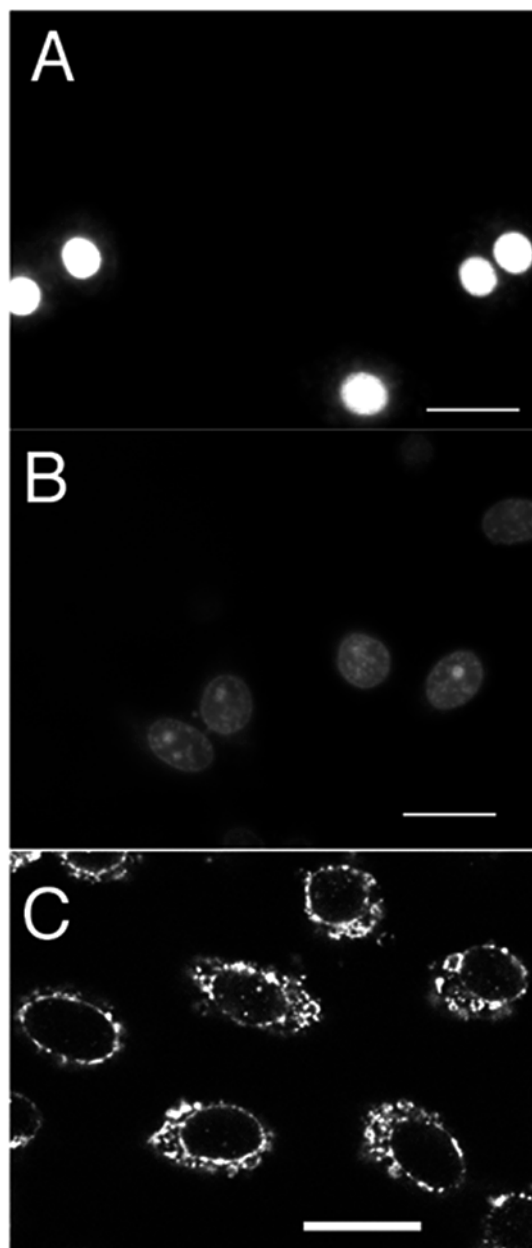


Fig. 2. The effect of rabbit reticulocyte lysate on transport of NLS-allophycocyanin and TR-DNA in digitonin-permeabilized HeLa cells. Epifluorescence images after 60 minutes exposure of the permeabilized cells to 20 µg/ml NLS-allophycocyanin with RRL (A) or without RRL (B). Confocal fluorescence image after 60 minutes exposure to 4 µg/ml TR-DNA with RRL (C). Bars, 25 µm.

access the nucleus in digitonin-treated cells. In contrast to TR-DNA, this polyanion could not cross the nuclear envelope and remained sequestered in the cytoplasm (Fig. 1B).

DNA nuclear transport is not augmented by cytoplasmic extract

The nuclear transport of NLS-containing proteins in digitonin-permeabilized cells is dependent on the presence of a number of soluble cytoplasmic proteins (Adam and Adam, 1994; Adam and Gerace, 1991; Gorlich et al., 1994; Melchior et al., 1993; Moore and Blobel, 1993, 1994; Paschal and Gerace, 1995). These proteins can be supplied by the addition of a transport-competent cytoplasmic extract (i.e. rabbit reticulocyte lysate, RRL). The inclusion of RRL in the import buffer greatly increased the nuclear transport of NLS-containing proteins such as NLS-allophycocyanin (Fig. 2A, B) or NLS-streptavidin (data not shown). In contrast, the inclusion of the RRL failed to augment the nuclear transport of TR-DNA and in fact appeared to decrease the nuclear accumulation of TR-DNA (Fig. 2C). These results indicate that the addition of soluble cytoplasmic factors is not required for the nuclear import of DNA.

The inability of the cytoplasmic extract to aid nuclear transport may be due to the presence of an inhibitor. One such inhibitory substance could be RNA present in the rabbit reticulocyte extract. The addition of 10 µg total pig spleen RNA completely inhibited TR-DNA nuclear uptake and cytoplasmic binding (data not shown). However, RNase-treated reticulocyte lysate also failed to aid nuclear transport of TR-DNA (data not shown).

The effect of nuclear transport inhibitors on DNA transport

Digitonin-permeabilized HeLa cells were exposed to 4 µg/ml 932 bp TR-DNA without RRL under various conditions that inhibit the transport of NLS-containing proteins (Jans and Hubner, 1996). Addition of the lectin wheat germ agglutinin (WGA) to the import reaction blocked import of the TR-DNA (Fig. 3A). Nuclear accumulation of 932 bp DNA was also inhibited when the cells were incubated without exogenous energy (Fig. 3B), treated with 5 mM NEM (Fig. 3C), or incubated at 4°C (Fig. 3D). However, these four inhibitory conditions all resulted in increased perinuclear staining (Fig. 3). Quantitative analysis revealed a fivefold decrease in the number of spots in the nuclei for WGA and no energy conditions, while NEM and cold-treated cells were inhibited more than 50-fold (Fig. 4). Statistical analysis indicated that the number of spots of the four treatment groups (4°C, WGA, NEM and No Energy) were significantly less ($P=0.0001$) than the number of spots in nuclei exposed only to TR-DNA (None). In addition, DNA import was inhibited by chelation of divalent cations with EDTA (data not shown).

These results contrast with the inhibition induced by RNA and dextran sulfate, which greatly reduced nuclear, perinuclear and cytoplasmic staining (data not shown).

TR-DNA remains intact during nuclear import

Further studies were done to consider the possibility that the accumulation of fluorescent signal in the nucleus was due to partial digestion of the 932 bp TR-DNA. Such degradation could have produced labeled molecules less than 40-60 kDa,

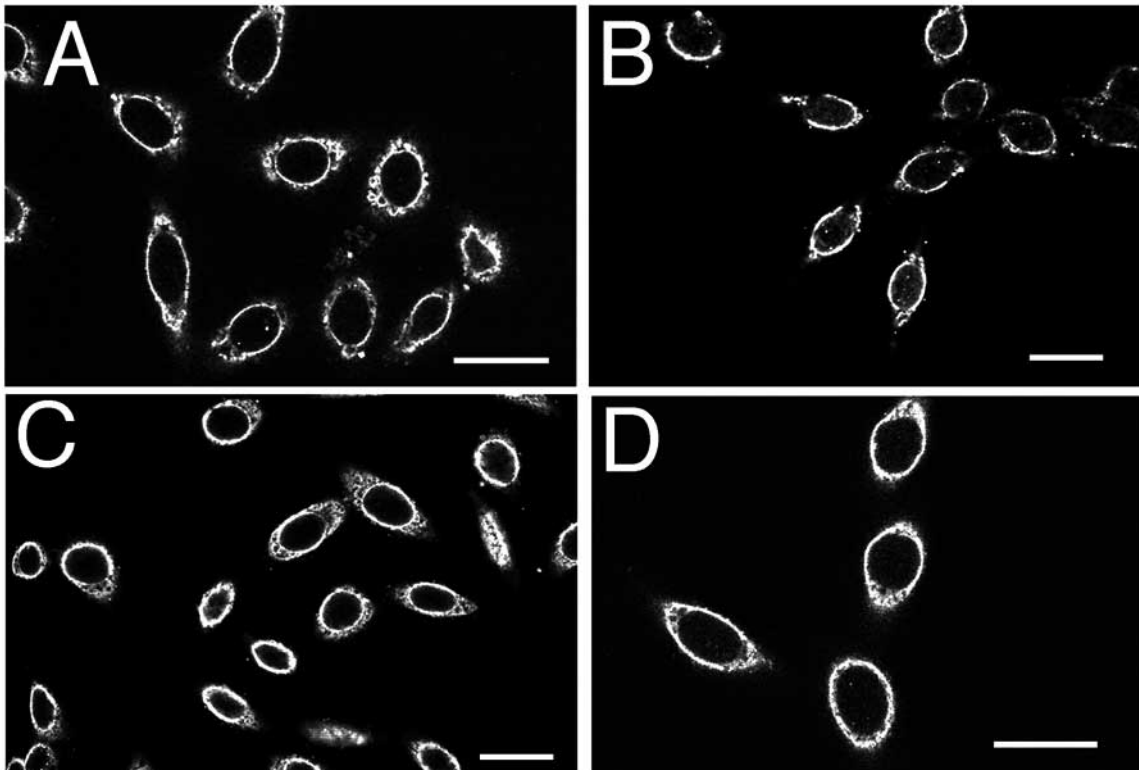


Fig. 3. Confocal fluorescence images of digitonin-permeabilized HeLa cells exposed for 60 minutes to 4 µg/ml TR-DNA without RRL under the following conditions: (A) WGA treatment (B) energy depletion, (C) NEM treatment and (D) 4°C. Bars, 25 µm.

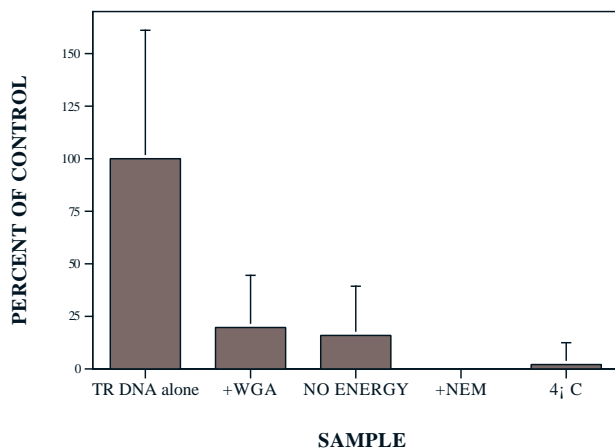


Fig. 4. A quantitative analysis of the images in Fig. 3 done on 25-30 nuclei for each type of treatment. The mean number of fluorescent spots in undamaged nuclei was compared to the mean value for nuclei exposed only to TR-DNA. T bars represent the standard deviation.

which would be below the size exclusion limit of the nuclear pore for diffusion (Paine, 1992). If DNA entry into the nucleus is the result of degradation of the TR-DNA to a diffusible size, then it would be expected that DNase-treated DNA would accumulate in the nucleus under similar conditions and with similar staining patterns. Instead, the addition of 1 unit of DNase I to the TR-DNA (30 minutes at 37°C) prior to inclusion in the import assay resulted in a change of the TR-DNA accumulation pattern from the characteristic punctate pattern to a diffuse pattern with nucleolar staining (Fig. 5A). On agarose gel electrophoresis the digested TR-DNA contained DNA fragments from 300 bp to less than 100 bp. (data not shown).

In addition, we assayed the import of a rhodamine-labeled 37-base oligonucleotide (molecular mass approx. 12 kDa) under TR-DNA transport conditions (import buffer without extract) and under conditions that inhibit TR-DNA accumulation in the nucleus. In contrast to the TR-DNA accumulation pattern of distinct punctate spots, rhodamine-oligonucleotide displayed a patchy, diffuse staining pattern and exhibited nucleolar accumulation (similar to that observed with DNase treated TR-DNA) (Fig. 5B). Cold treatment (4°C), NEM treatment, energy depletion and WGA treatment all failed to inhibit rhodamine-oligonucleotide transport to the nucleus (data not shown), which is consistent with previous studies involving microinjected labeled oligonucleotides (Chin et al., 1990; Leonetti et al., 1991). These results suggest that the pathway for the nuclear transport of rhodamine-labeled 37-base oligonucleotide is different from that of 932 bp TR-DNA.

Direct evidence indicating that the DNA remained intact throughout nuclear transport was also obtained. Southern blot analysis was performed on DNA extracted from nuclei that were isolated from digitonin-permeabilized cells exposed to the 932 bp PCR DNA under optimal conditions for transport (no extract) (Fig. 6). No degradation of the PCR DNA was observed either under the optimal condition when intranuclear DNA is observed (Fig. 6, lane 2) or with WGA treatment when no intranuclear DNA is found (Fig. 6, lane 3). The amount of DNA associated with the nuclei was similar under both con-

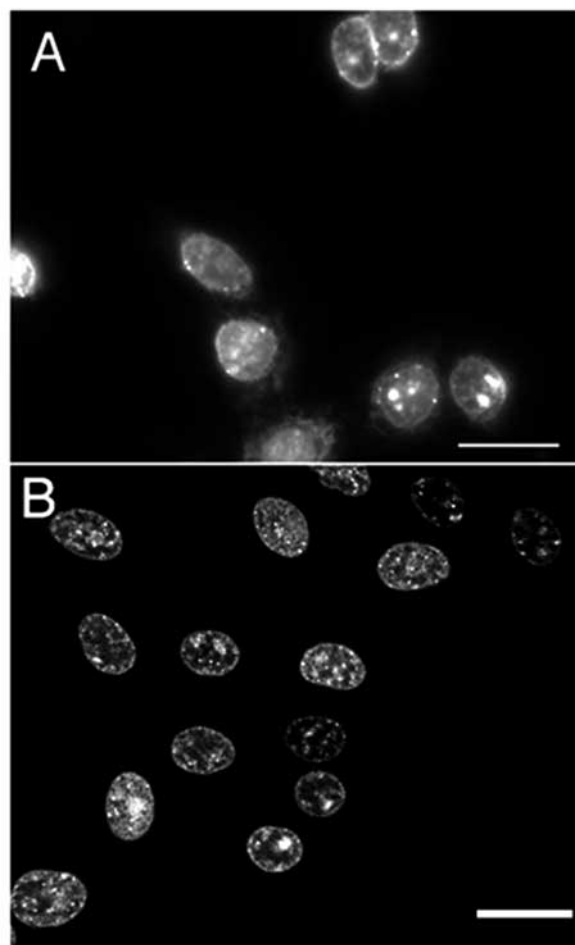


Fig. 5. Digitonin-permeabilized HeLa cells in import buffer without RRL were exposed at 37°C for 60 minutes to either: (A) 16 µg/ml of DNase-treated 932 bp TR-DNA or (B) 10 µg/ml of a rhodamine-labeled 37-base oligonucleotide. Bars, 25 µm.

ditions because the isolated nuclei contain both perinuclear and intranuclear PCR DNA. While the WGA-treated nuclei have substantially less intranuclear PCR DNA than the untreated nuclei, they still have substantial amounts of perinuclear DNA (Fig. 3A).

TR-DNA import into the nucleus is size dependent

The import assay was performed using larger PCR fragments to assess the effect of DNA size on nuclear transport. In

Fig. 6. Condition of the 932 bp PCR DNA after Southern blot analysis of nuclear-associated DNA following a 45 minute nuclear import in extract-free, digitonin-permeabilized HeLa cells in import buffer. Lane 1, no PCR DNA added; lane 2, 932 bp PCR DNA; lane 3, 932 bp PCR DNA + WGA treatment. Arrow indicates the migration point of the 932 bp PCR DNA.

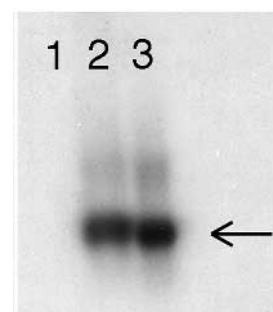
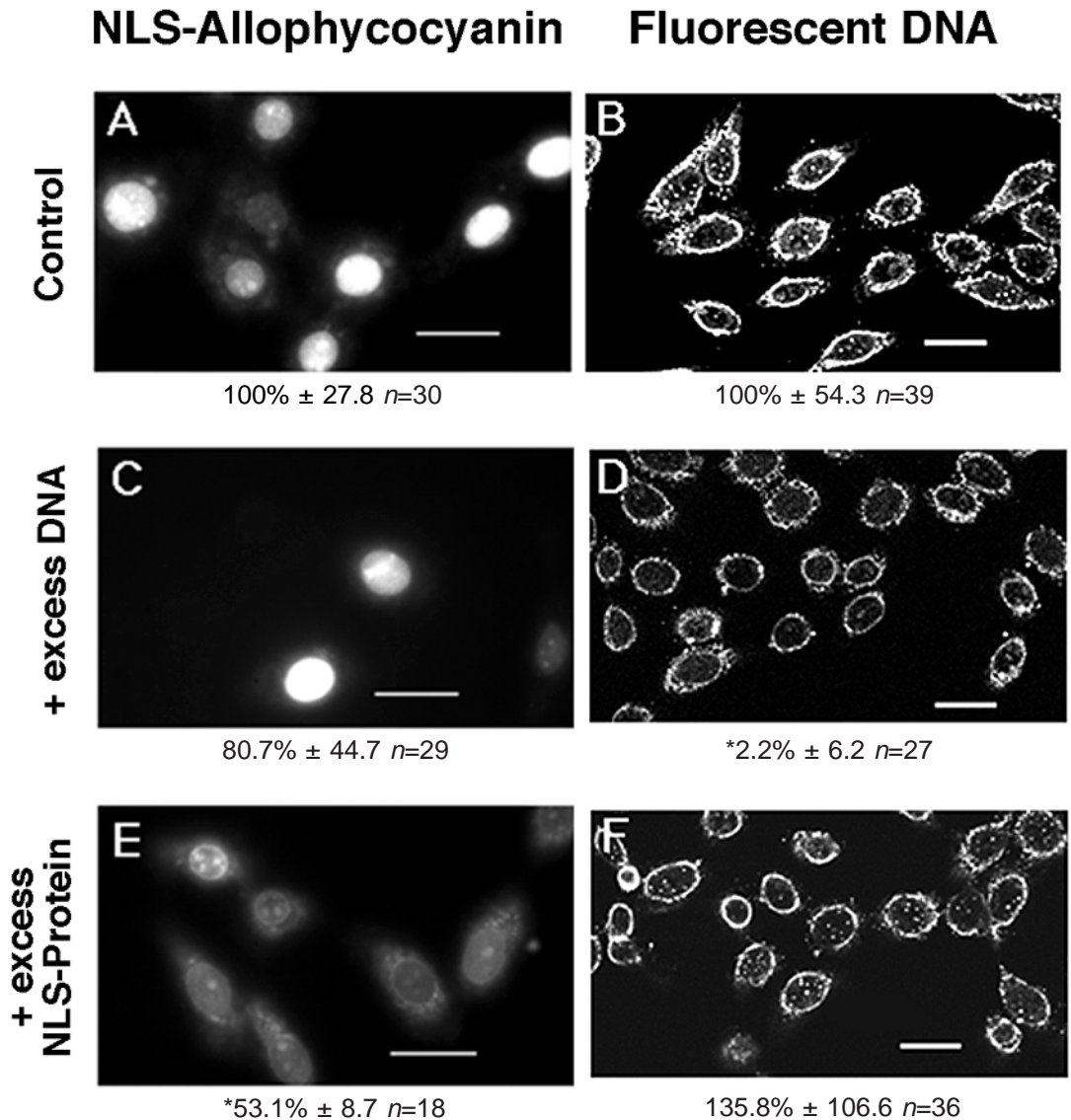


Fig. 7. The effect of excess DNA on NLS-protein transport and vice versa. Fluorescent images of digitonin-permeabilized HeLa cells exposed for 60 minutes in a transport assay to the following transport substrates: (A) 20 $\mu\text{g/ml}$ NLS-allophycocyanin; (B) 4 $\mu\text{g/ml}$ fluorescein-DNA (Fl-DNA); (C) 20 $\mu\text{g/ml}$ NLS-allophycocyanin and 70 $\mu\text{g/ml}$ unlabeled 932 bp PCR DNA; (D) 4 $\mu\text{g/ml}$ Fl-DNA + 70 $\mu\text{g/ml}$ unlabeled 932 bp PCR DNA; (E) 20 $\mu\text{g/ml}$ NLS-allophycocyanin and 400 $\mu\text{g/ml}$ NLS-streptavidin; (F) 4 $\mu\text{g/ml}$ Fl-DNA + 400 $\mu\text{g/ml}$ NLS-streptavidin. For A,C,E (epifluorescence), mean pixel values ($\pm\text{s.d.}$) of undamaged nuclei were compared to the mean value for nuclei exposed only to NLS allophycocyanin (control in A). For B,D,F (confocal fluorescence), mean number of fluorescent spots ($\pm\text{s.d.}$) of middle slices of undamaged nuclei were compared to the mean value for nuclei exposed only to DNA (control in B). *n*, the number of nuclei examined; *, significant difference between experimental and control ($P=0.0001$). Bars, 25 μm .



contrast to the staining observed with the 932 bp fragment, the 2 kb, 3 kb and 5 kb DNAs exhibited mainly cytoplasmic and perinuclear staining, with approximately 5% of the cells containing intranuclear, fluorescent spots (data not shown).

TR-DNA and NLS-proteins do not competitively inhibit each other

The fact that TR-DNA was not dependent on exogenous soluble transport factors for nuclear localization, while NLS-containing proteins were dependent, raised the question of whether they enter the nucleus using different nuclear pore mediators. It has previously been shown that it is possible to competitively inhibit the transport of labeled NLS-proteins with excess unlabeled NLS-proteins (Breeuwer and Goldfarb, 1990). Excess unlabeled 932 bp DNA did not inhibit NLS-allophycocyanin nuclear import (Fig. 7C), but did inhibit both nuclear and cytoplasmic accumulation of labeled DNA (Fig. 7D). Control experiments demonstrated that import of NLS-allophycocyanin was inhibited by excess NLS-streptavidin (Fig. 7E). In the reciprocal experiment, TR-DNA nuclear

uptake was not affected by incubation with excess (about 20-fold) NLS-allophycocyanin (Fig. 7F).

TR-DNA is not efficiently transported to HeLa cell nuclei after microinjection

HeLa cells were microinjected with 100 $\mu\text{g/ml}$ of 932 bp TR-DNA. While perinuclear accumulation of the injected DNA was apparent in many of the cells, none of them accumulated intranuclear-labeled DNA by 4 hours after injection (Fig. 8). Also, the labeled DNA did not enter nuclei by 24 hours after microinjection (data not shown).

DISCUSSION

We have found that double-stranded DNA, approximately 1 kb in length, enters the majority of nuclei in digitonin-permeabilized cells (Fig. 1A). Most of the nuclear DNA was located in several intense non-nucleolar spots with a small amount of diffuse nuclear distribution. The present study and previous

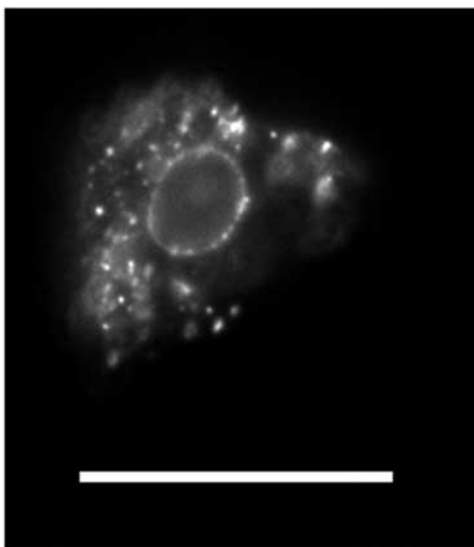


Fig. 8. HeLa cells were cytoplasmically microinjected with 100 $\mu\text{g/ml}$ TR-DNA and epifluorescence images were obtained after 4 hours. Bar, 25 μm .

work indicate that the integrity of the nuclear membrane is not compromised under these conditions (Adam et al., 1991). The nuclear uptake was neither dependent upon the DNA sequence nor the fluorescent label, and was also observed in several types of digitonin-permeabilized cells. The ability of DNA molecules to enter intact nuclei in permeabilized cells is consistent with the ability of microinjected DNA to enter the post-mitotic nuclei of myotubes in culture (Dowty et al., 1995).

Southern blot analysis indicated that the nuclear-associated DNA was not degraded (Fig. 6). Given that the nuclear-associated DNA is composed of perinuclear, intranuclear, and perhaps even contaminating cytoplasmic DNA, the Southern blot analysis may not have detected a small amount of degraded DNA that accounts for the intranuclear staining. However, several other experiments indicate that nuclear import of TR-DNA is not due to diffusion of a degradation product through the nuclear pore complex. Inhibition of import by depletion of energy or cold treatment in the import assay (Fig. 3) shows that DNA import is an active process. A comparison of the transport properties of a TR-labeled oligonucleotide with 1 kb TR-DNA clearly shows a distinction between the active transport of intact DNA and the passive diffusion of a small molecule. None of the conditions which inhibit intact DNA transport (energy depletion, WGA treatment, cold treatment or NEM treatment) affect the diffusion of oligonucleotides into the nucleus. The patchy, diffuse pattern of oligonucleotide accumulation in the nucleus is quite different from the distinct punctate pattern observed following transport of intact TR-DNA (Fig. 5). Also, the transport of DNase-treated DNA resembled that of oligonucleotides (Fig. 5A).

Some information about the mechanism by which DNA enters the nucleus can be derived from the conditions under which transport is inhibited. WGA inhibited the nuclear entry of DNA in the present study and in our previous microinjection study (Dowty et al., 1995). WGA binds *N*-acetyl glucosamine groups present on nuclear pore proteins and inhibits

the facilitated transport of both proteins and RNA through the pore (Dabauvalle et al., 1988; Finlay et al., 1987; Yoneda et al., 1987). The inhibition by WGA suggests that DNA entry is also via the nuclear pore. This is consistent with our previous observation that microinjected gold-labeled DNA was occasionally located at the nuclear pore (Dowty et al., 1995).

In contrast to the nuclear import of karyophilic proteins in this system, the import of the DNA did not require the addition of cytoplasmic extract, which contains NLS receptors and the GTPase Ran/TC4 (Adam and Adam, 1994; Adam et al., 1991; Moore and Blobel, 1993). This would suggest that the nuclear transport of DNA either does not require these transport factors or requires much less of them. The fact that NLS-proteins and DNA did not compete for nuclear entry also suggests that these cytoplasmic factors are not rate-limiting for DNA nuclear entry. On the other hand, the inability of the cytoplasmic extract to aid nuclear transport may be due to the presence of an inhibitor. Our future studies on the inhibitory effect of cytoplasmic extract on DNA transport and the expanding discoveries of new NLS-receptors and other co-factors will enable the role of cytosolic factors in DNA transport to be better defined (Rout et al., 1997).

Perhaps DNA import is aided by interactions with nuclear pore proteins such as nup358 (RanBP2) and nup153, which contain zinc finger domains that bind DNA (Sukegawa and Blobel, 1993; Wu et al., 1995; Yokoyama et al., 1995). The several nucleoporins that bind RNA through a conserved amino acid motif could also bind DNA (Fabre et al., 1994). RNA and dextran sulfate may block DNA nuclear transport by competing for such binding sites in the nuclear pore. The fact that these polyanions also block cytoplasmic localization of labeled DNA raises the possibility that polyelectrolyte interactions within cytoplasmic elements may also play a role in DNA uptake. The inability of another polyanion such as dextran sulfate to enter nuclei suggests that DNA has additional features that enable nuclear transport.

The nuclear transport of other karyophilic molecules has been divided into two steps: nuclear pore docking and translocation through the pore. A similar two-step process could also be the model for DNA nuclear transport. The RNA, dextran sulfate, and excess unlabeled DNA may block DNA transport by inhibiting DNA docking at the pore. The WGA, NEM, cold or energy depletion conditions could cause the perinuclear staining by inhibiting the translocation of DNA docked at the nuclear pore (Fig. 3). Electron microscopic studies are required to determine the more precise sub-cellular location of the DNA under these various inhibitory conditions.

Some components of the NLS-protein import machinery have been shown to be sensitive to sulfhydryl alkylation by NEM (Chi et al., 1995). The presence of insoluble components of the protein import machinery that are NEM-sensitive could explain NEM inhibition of DNA transport in the absence of cytoplasmic extract (Adam and Gerace, 1991). It has been reported that glycosylated proteins enter the nuclei of digitonin-permeabilized cells without the aid of cytosolic proteins (Duverger et al., 1993, 1995). However, in contrast to DNA nuclear transport, their transport was not NEM-sensitive.

In digitonin-permeabilized cells, DNA greater than 2 kb remained in the cytoplasm and perinuclear area, and entered the nucleus with less efficiency than 1 kb DNA. This may be because a larger DNA molecule is more likely to become

sequestered outside the nucleus because it binds more cytoplasmic elements. A critical ratio of DNA binding sites inside and outside the nucleus could determine whether or not nuclear transport will occur. Another explanation is that a very long piece of DNA (more than 2 kb) would be more likely to bind several pores, inhibiting import. The distance between nuclear pores, which varies with cell type and metabolic activity, ranges from about 0.1 μm to 0.6 μm (3 to 80 pores per square μm of nuclear membrane) (Stewart, 1992). This distance is comparable to the length of a piece of DNA of approx. 2 kb (about 0.6 μm). In addition, translocation across a nuclear pore may be less efficient for the larger DNA. The decreased efficiency of nuclear uptake by the larger-sized DNA provides additional evidence that the uptake of the 932 bp DNA was not due to DNA degradation.

Fluorescently labeled 932 bp DNA does not accumulate to visible quantities in the nucleus of microinjected HeLa cells. Similarly, biotinylated plasmid DNA (approx. 6 kb) could not be visualized within nuclei after microinjection into the cytoplasm of myotubes (Dowty et al., 1995). Plasmid DNA containing a reporter gene can be expressed in microinjected cells but with relatively poor efficiency (Brinster et al., 1985; Capecchi, 1980; Dowty et al., 1995; Mirzayans et al., 1992; Thorburn and Alberts, 1993). Since the nuclear entry of only one or a few DNA molecules can enable foreign gene expression, the efficiency of DNA nuclear transport required for expression in a living cell may be below the threshold required for direct detection using fluorescently labeled DNA. Furthermore, it may be difficult to compare the microinjection and permeabilized cell results since much more DNA is delivered in the permeabilized cells. Another difference between microinjection and permeabilization is that digitonin could disrupt insoluble cytoplasmic elements (Cook et al., 1983). However, this has not been observed at the concentration used in this study (Fiskum et al., 1980). In addition, the poor efficiency of DNA nuclear transport in the intact cell could be due to soluble cytoplasmic factors that inhibit the nuclear uptake of microinjected DNA but diffuse out of the digitonin-permeabilized cell. The partial inhibition of DNA uptake by the addition of cellular extract to permeabilized cells (Fig. 2C) is consistent with this hypothesis and provides an approach for elucidating these factors.

In summary, translocation of DNA across the nuclear membrane is similar to that of NLS-containing proteins in that it requires energy, utilizes NEM-sensitive proteins and is inhibited by WGA. However, the nuclear transport of DNA is different from that of other macromolecules in that it does not require the addition of cytoplasmic soluble factors for localization to or transport across the nuclear membrane. These results have important implications for the design of new gene transfer vectors and need to be incorporated into hypotheses of how viral nucleic acids are transported to the nucleus. As has been previously proposed for simian virus 40 and adenoviral DNA nuclear transport, viral proteins may enable nuclear membrane targeting of the nucleic acid and avoidance of cytoplasmic sequestration (Clever et al., 1991; Greber et al., 1993). Once at the nuclear pore, the viral DNA would not need an NLS in order for it to enter the nucleus.

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