INTRODUCTION

The transport of DNA into the nucleus of mammalian cells is of importance to the study of DNA viruses such as adenovirus and Herpes virus (Sebestyén et al., 1998; Whittaker and Helenius, 1998; Kasamatsu and Nakanishi, 1998) and the development of non-viral vectors for gene therapy. A cornerstone of these efforts is the tremendous advances in our understanding of how proteins and RNA are transported in and out of the nucleus (Mattaj and Englmeier, 1998; Pennisi, 1998). These macromolecules contain (or bind to proteins that contain) nuclear localization signals (NLSs) or export signals that bind to nuclear transport proteins (e.g. karyopherins $\alpha$ and $\beta$), which mediate their directional movement through the nuclear pore. Viral proteins also contain NLSs and the exact mechanism by which they enable DNA nuclear entry is under active study.

A variety of non-viral methods of gene transfer are under development for gene therapy (Wolff, 1994; Wolff and Trubetskoy, 1998). The therapeutic gene, usually in the form of a plasmid DNA, must be transported from the exterior of the cell to the nucleus in order for it to be expressed and have its healing effect. While naked DNA (linear or plasmid) can traverse the nuclear pores of intact nuclei (Dowty et al., 1995; Hagstrom et al., 1997), the process is inefficient as compared to that of viral genomes and karyophilic proteins. A manifest approach to increase the efficiency of foreign DNA nuclear entry is to link the DNA with an NLS. Several groups have reported that the non-covalent association of an NLS with DNA can enhance DNA nuclear transport (Collas and Alestrom, 1997; Dean, 1997; Kaneda et al., 1989; Langle-Rouault et al., 1998). Our laboratory has found that while the non-covalent association of an NLS with plasmid DNA (using another method) can increase its efficiency of expression, the mechanism was not by increasing DNA nuclear entry (Fritz et al., 1996). Inferring that the NLS has to be tightly associated with the gene, we developed a new approach for covalently attaching NLSs to DNA (Sebestyén et al., 1998). The covalently-attached NLS was functional in the digitonin-treated cell system but was unable to enhance DNA nuclear entry in living cells after microinjection.

SUMMARY

Although the entry of DNA into the nucleus is a crucial step of non-viral gene delivery, fundamental features of this transport process have remained unexplored. This study analyzed the effect of linear double stranded DNA size on its passive diffusion, its active transport and its NLS-assisted transport. The size limit for passive diffusion was found to be between 200 and 310 bp. DNA of 310-1500 bp entered the nuclei of digitonin treated cells in the absence of cytosolic extract by an active transport process. Both the size limit and the intensity of DNA nuclear transport could be increased by the attachment of strong nuclear localization signals. Conjugation of a 900 bp expression cassette to nuclear localization signals increased both its nuclear entry and expression in microinjected, living cells.

Key words: Nuclear localization signal, Expression, DNA transport
extended form of the SV40 T antigen NLS (NLS/NL) was used since it was found to be more efficient for the nuclear transport of large macromolecules such as IgM (Hubner et al., 1997; Rihs et al., 1991; Yoneda et al., 1992). Our results show that this NLS/NL is able to enhance the nuclear uptake of DNA both in digitonin treated and microinjected cells in a size-dependent way. The tight association of NLS/NL with a 900 bp DNA increased both its physical entry into the nucleus and expression of an encoded marker gene, a finding with therapeutic implications.

MATERIALS AND METHODS

DNA production

Fluorescein-labeled oligonucleotides were purchased from and PAGE purified by Integrated DNA Technologies (Corvalle, IA). DNAs of different sizes were produced by using the polymerase chain reaction (PCR) to amplify a small region of lambda DNA. 78, 118, 200, 318, 518 and 997 base pair (bp) PCR products were produced by using one common primer (5' GAC GGA TTC AAC ATG CTA AAC AGT GTT AAA TTA GAA A 3') and a primer specific for each individual size: 78 bp (5' GAC GGA TCT TTT GTT ATT ATG ATG ACC AGT TCT A 3'), 118 bp (5' GAC GGA TCT TTT GCAG GAG ATT ATT T 3'), 1500 bp (5' ATACCAGAAGATCTGAGATGCTGCCTA 3'), 2000 bp (5' ATCCAGAAGATCTGAGATGCTGCCTA 3'), and 997 bp (5' GAC GGA TCT TTT GTT ATT ATG ATG ACC AGT TCT A 3'). Both primers had a HindIII restriction site at the 5' end. The reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 25 mM dNTP (Pharmacia, Piscataway, NJ), 0.8 mM of each primer, 0.2 pmol template, and 2.5 units of Taq polymerase (Promega, WI) per 100 μl of PCR reaction. Cycling parameters were calculated based on template length and annealing temperature of the primers. Purification of amplified labeled-DNA consisted of washing on a Millipore Ultrafree-MC filter (30 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) diluted 10-fold. Samples were washed 5-10 times (a minimum of 2,500 fold dilution of contaminants), and selected filtrates were checked for the presence of unincorporated fluorescent oligonucleotides using both agarose gel electrophoresis and spectrofluorometry.

In order to produce DNA with biotin at one end only, the common primer above (with the BamHI site) was used with primers specific for 518 bp (5' GAC GGT ACC TTT CTT GTA GTA ATC ACT TCA CTC A 3'), 997 bp (5' GAC GGT ACC TTT CTT GTA GTA ATC ACT TCA CTC A 3') and 1500 bp (5' GAC GGT ACC TTT CTT GTA GTA ATC ACT TCA CTC A 3'). The Klenow was inactivated by a 10 minute incubation at 75°C. Unincorporated nucleotide was removed by washing the DNA 5-10 times (at least 2,500-fold dilution of the contaminant) on a Millipore Ultrafree-MC filter (30 kDa cut-off) in 'base import buffer'. A small aliquot of DNA was assayed for biotin incorporation by binding to 5 μg of streptavidin. 80-100% of the biotin-labeled DNA was shifted on an ethidium bromide stained 2% agarose gel.

DNAs with one BamHI site and one Acc65I site were digested with BamHI as above, but filled in with 10 μM biotin dATP, dCTP, dGTP and dTTP. This DNA was then digested with Acc65I and filled with 10 μM Texas Red dCTP, dTTP, dATP and dGTP. This produced DNA with a biotin on one end and a Texas Red molecule on the other. 70-90% of the biotin-labeled DNA was shifted when assayed for streptavidin binding on an ethidium bromide stained 1% agarose gel.

In order to equalize the number of fluorochromes on a per weight basis, some DNAs were labeled by incorporating fluorescent nucleotides during the PCR reaction as previously described (Hagstrom et al., 1997).

The 900 bp green fluorescent protein (GFP) expression vector consisted of a minimal CMV promoter (~117 to +17 bp in relation to the transcriptional start site of the human immediate early cytomegalovirus promoter obtained from base pairs 619-753 of the pCl mammalian expression vector; Promega, WI) 5' to the complete, 717-bp S65T GFP coding sequence (Clontech), which was followed by a minimal polyA signal sequence (AAAT AAA AGA TTA AAT TTA TTA TTG GGT GGT GGT TTT TTG TG) (Briggs and Proudfoot, 1989). Production and labeling of this vector was by PCR using a biotin-labeled 5' primer (5' GCA CCA AAA TCA ACG GGA TTC CTT AAA ATG T 3') and a Cy5-labeled 3' primer (5' CAC ACA AAA AAA CCA ACA CAC AGA TCT AAT GA 3').

Streptavidin-peptide conjugation

Streptavidin was covalently conjugated as previously reported (Adam et al., 1992) to either a 39 amino acid peptide (H-CKKKSSSSDEATDSQHSTTPKKKRVEDPKDFPSLELS) containing a functional SV40 large T antigen NLS (Kalderon et al., 1984) (streptavidin-NLS/NL), or to a mutant version of it that has a K to T amino acid change at position 24 (corresponding to the K25 position of the SV40 T antigen, known to be transport deficient; Kalderon et al., 1984). The mutant peptide also has an additional K near the amino terminus at position 2 (H-CKKKSSSSDEATDSQHSTTPKKKRVEDPKDFPSLELS) (streptavidin-NLS/MNL) to keep the overall charge of the two peptides equal. The average number of peptide molecules per streptavidin monomer was estimated to be 2 (and thus, 8 for the streptavidin tetramer) by analysis of mobility shift on SDS-polyacrylamide gel electrophoresis.

Streptavidin-NLS/NL or streptavidin-NLS/MNL was bound to fluorescein biotin at a ratio of 50:1 on ice for 30 minutes. Removal of unbound fluorescein biotin was accomplished by gel filtration on a NAP 5 column (Pharmacia).

Streptavidin-NLS/NL or streptavidin-NLS/MNL was added to biotinylated DNA at a molar excess of between 2 and 10.

Transport assay

Transport assays were performed as previously described (Adam et al., 1992; Hagstrom et al., 1997) for one hour. Omission of ‘energy’ in a transport assay consisted of replacing the ATP, GTP, creatine phosphate, and creatine phosphokinase with buffer. Wheat germ agglutinin (WGA) treatment involved incubating the digitonin-treated cells in import buffer containing 0.16 mg/ml WGA for 10 minutes at room temperature and including 1 mg/ml WGA in the transport solution.

Microinjection

HeLa cells grown on untreated glass coverslips were microinjected in the cytoplasm using an IM 200 microinjector (Narishige, Tokyo) (Dowty et al., 1995). All microinjected materials were co-injected with 1 mg/ml of either 580 kDa fluorescein-labeled dextran (Sigma) or 500 kDa Cascade Blue-labeled dextran (synthesized by Vladimir Budker). The microinjected cells were fixed in 2% formaldehyde and
mounted in Gel/Mount. Nail polish was used to create a raised border on the slide to prevent shearing of the cells.

**Microscopy and quantitation**

Images of the samples were collected by epifluorescence microscopy on a Nikon Optiphot with a SenSys CCD Camera (Photometrics, Tucson, AZ) using a ×100 oil plan apo objective with NA 1.4. In our previous publication (Hagstrom et al., 1997), confocal microscopy was used to determine the subcellular location of fluorescent DNA. Based on comparisons of samples analyzed by both confocal microscopy and epifluorescent microscopy, and given the large size of the HeLa cell nuclei, it was determined that epifluorescence microscopy was sufficient to ascertain whether the labeled DNA was intranuclear.

When DNA is end labeled with one or two fluorochromes, different size DNAs will have the same number of fluorochromes per DNA molecule but different amounts of label per μg. Thus it was problematic to perform direct quantitative comparisons among the different DNA sizes. So, to compare different sized DNAs, qualitative analysis of the cytoplasmic signal relative to the nuclear signal on an individual cell basis was used to judge the efficiency of nuclear accumulation. Unless otherwise indicated, the photomicrographs in the figures show representative examples.

To compare DNAs of the same size, quantitative analyses were used to compare the ratio of DNA transport with and without streptavidin-NLS/NL (Fig. 5O). Mean pixel values in the center of nuclei were determined and background values were subtracted. Background-subtracted mean pixel values were averaged within groups and their standard deviations calculated. Two-sample t-tests, without the assumption of equal variances, were applied to the mean pixel values. In order to protect against potential problems with non-normality, the two-sample Wilcoxon test was also computed. If the Wilcoxon P-value was larger than the t-test P-value, the Wilcoxon P-value was used. Otherwise, the t-test P-values were used. The averages for NLS samples were divided by the averages for non-NLS samples to obtain a ‘+ to –’ ratio seen in Fig. 5O. The standard error of the ratio was calculated using the delta method (Agresti, 1990).

**RESULTS**

**Nuclear accumulation of DNA is size dependent**

Linear DNA fragments of various sizes were labeled with Texas Red fluorophores and assayed for their ability to accumulate in the nuclei of digitonin-permeabilized HeLa cells in the absence of rabbit reticulocyte lysate (RRL) (Fig. 1). Our previous study showed that linear DNA of ~1 kb in size is transported into the nucleus by an active process that is inhibited by RRL (Hagstrom et al., 1997). Seventy bp DNA accumulated strongly in nucleoli and exhibited diffuse staining throughout the rest of the nucleus, with occasional punctate nuclear staining (Fig. 1A). The 110 and 200 bp DNAs distributed similarly to 70 bp DNA, but had additional small punctate accumulation in the non-nucleolar regions of the nucleus (Fig. 1B,C). 310 bp and 510 bp DNA showed binding to the nuclear membrane, and exhibited large and frequent punctate staining in the nucleus with no nucleolar staining (Fig. 1D,E). The 1 kb DNA sample also had punctate DNA staining in the nuclei (Fig. 1F) which was decreased as compared to the 510 bp DNA specimen (Fig. 1E). The 1.5 kb DNA sample exhibited smaller and fewer nuclear spots (Fig. 1G), while the 2 and 3 kb DNAs had no punctate staining and minimal diffuse staining (Fig. 1H,I).

The ability of the different sized DNAs to enter the nuclei of digitonin-permeabilized cells was also analyzed in the presence of RRL and ‘energy’ at 37°C (see Fig. 5). The addition of RRL did not prevent the nuclear accumulation of the 70 to 200 bp DNAs (Fig. 5A,C,E) but inhibited the punctate nuclear staining from the DNAs greater than 310 bp (Fig. 5F,H,J,L,N) as previously noted for 1 kb DNA (Hagstrom et al., 1997).

The DNAs of different sizes were microinjected along with fluorescently-labeled dextran (580 kDa) into the cytoplasm of HeLa cells and harvested after about 4 hours (Fig. 2). Only the...
cells in which the dextran was excluded from the nuclei were included in the analyses. The 70 and 110 bp DNA accumulated as a non-nucleolar haze in the nuclei (Fig. 2A,C). The 200 bp DNA exhibited both a punctate nuclear accumulation as well as a non-nucleolar haze (Fig. 2E,F). The 310 bp DNA sample exhibited less nuclear accumulation (Fig. 2F) while 510 bp and 1 kb DNA samples had very little (Fig. 2H,J). DNAs of 1.5, 2 and 3 kb size were unable to efficiently enter nuclei under these conditions (data not shown).

**DNA can passively enter intact nuclei in a size-dependent manner**

The DNAs of different sizes labeled with Texas Red fluorophores were also assayed for their ability to enter the nuclei of digitonin-treated cells at 4°C with neither 'energy' nor RRL. The 70 bp DNA accumulated evenly throughout nuclei (Fig. 3A), while 110 bp DNA displayed both hazy and small, punctate staining (Fig. 3B,C). The 200 bp DNA also exhibited this pattern (Fig. 3D). The 310 bp DNA had a very small level of nuclear haze and little of the punctate staining (Fig. 3D,E) that was prominent at 37°C and with ‘energy’ (Fig. 1D-F). Nuclear rimming was more prominent in nuclei exposed to DNA greater than 310 bp (Fig. 3). These results indicate that there is a transition in the accumulation efficiency of 200 and 310 bp double-stranded DNA at 4°C.

**Fig. 2.** Effect of DNA size on sub-cellular distribution and nuclear accumulation pattern in cells at 37°C about 4 hours after cytoplasmic injection. 70 bp (A), 70 bp + streptavidin-NLS/NL (B), 110 bp (C), 110 bp + streptavidin-NLS/NL (D), 200 bp (E), 310 bp (F), 310 bp + streptavidin-NLS/NL (G), 510 bp (H), 510 bp + streptavidin-NLS/NL (I), 1 kb (J), 1 kb + streptavidin-NLS/NL (K). 220 ng/µl - 1.75 µg/µl DNA labeled at both ends with one Texas Red fluorophore (A-D and F), at one end with one Cy5 fluorophore (E), or at one end with one Texas Red fluorophore (G-K) was microinjected. Representative examples of transporting cells are displayed.

**Fig. 3.** Effect of DNA size on sub-cellular distribution and nuclear accumulation pattern in digitonin-permeabilized cells at 4°C with neither ‘energy’ nor RRL. 70 bp (A), 110 bp (B), 200 bp (C), 310 bp (D), 510 bp (E) and 1 kb (F). 200 ng DNA labeled by PCR incorporation of Texas Red fluorophores (A-B and D-E), or by Cy5-labeled primer incorporation (C). Representative examples are displayed.
The effect of DNA size on NLS-mediated transport in digitonin-treated cells

The following experiments were conducted to determine the effect of DNA size on the nuclear entry of DNA conjugated to a karyophilic protein. DNAs of various sizes were labeled with a Texas Red fluorophore and a biotin at one or each end. The DNA was mixed with streptavidin containing various covalently-linked NLSs. If the NLS was the canonical SV40 T ag NLS (PKKKRKV) (Yoneda et al., 1992), the DNA aggregated because of charge interactions between the negatively-charged DNA and the positively-charged NLSs (data not shown). When streptavidin containing covalently-attached NLS/NL (streptavidin-NLS/NL) or a non-functional NLS (streptavidin-NLS/MNL) was mixed with linear DNA containing a single biotin, more than 90% of the DNA was shifted from its original position when assayed by agarose gel electrophoresis (Fig. 4; compare lane 2 to lane 3 and 4, arrow A). Biotinylation had no effect on the migration of the DNA (data not shown). Aggregation was not observed with streptavidin-NLS/NL or NLS/MNL (Fig. 4) because it contains an extended form of the SV40 T ag NLS (labeled NLS/NL) that is less positively charged (+1.5). On the basis of size, it appeared that the streptavidin complexes mostly contained one or two DNA molecules (Fig. 4; lanes 3 and 4, arrows B and C, respectively).

The complexes of streptavidin-NLS/NL and biotinylated DNA of various sizes were applied to digitonin-permeabilized cells in the presence of RRL and ‘energy’ at 37°C (Fig. 5). With streptavidin-NLS/NL, biotinylated DNA (up to 3 kb) accumulated in the nucleus. 70 bp and 110 bp DNA accumulated both with and without streptavidin-NLS/NL present (Fig. 5A-D). DNA of sizes 310 bp and larger all formed punctate staining in the presence of streptavidin-NLS/NL (Fig. 5G,I,K,M,O), while virtually no nuclear accumulation occurred without streptavidin-NLS/NL (Fig. 5F,H,J,L,N). The ratios of mean pixel values of NLS-mediated to non-NLS-mediated transport are shown in Fig. 5P. All DNA sizes tested exhibited a significant (P < 0.0002) enhancement of transport (at least 2.5-fold). When the 510 bp biotinylated DNA was bound to streptavidin-NLS/MNL, it was not able to enter nuclei (data not shown). Without DNA, streptavidin-NLS/NL entered the nucleus while streptavidin-NLS/MNL did not (data not shown).

SDS/PAGE analysis indicated that the modified streptavidin used in the experiments contained on average two peptides per streptavidin monomer (data not shown) and therefore, eight peptides per streptavidin tetramer. If the streptavidin monomer contained less than two peptides, DNA nuclear import was less efficient (data not shown).

Wheat germ agglutinin (WGA) was used to determine whether the enhanced uptake of the complexes of streptavidin-NLS/NL and DNA was mediated via the nuclear pore (Forbes, 1992; Garcia-Bustos et al., 1991). Complexes containing DNAs ranging in size from 70 bp to 510 bp were incubated with digitonin-permeabilized cells with and without WGA. WGA prevented the DNA of all sizes from entering the nuclei resulting in an increased signal at the nuclear rim (data not shown). The intensity of nuclear fluorescence with WGA was significantly less (P ≤ 0.007) than the fluorescence without WGA for all sizes of DNA tested (data not shown).

Nuclear transport of DNA complexes with streptavidin-NLS/NL in living cells

The complexes of streptavidin-NLS/NL and DNA of various sizes were microinjected into the cytoplasm of HeLa cells and contained less than two peptides, DNA nuclear import was less efficient (data not shown).

Table 1. GFP expression in microinjected cells

<table>
<thead>
<tr>
<th>Streptavidin</th>
<th>Total cells</th>
<th>GFP+/DNA+</th>
<th>GFP+/DNA−</th>
<th>GFP−/DNA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLS/NL</td>
<td>272</td>
<td>11/11</td>
<td>8/0</td>
<td>12/4</td>
</tr>
<tr>
<td>(percentages)</td>
<td></td>
<td>(4.0)/(4.0)</td>
<td>(2.9)/(0)</td>
<td>(4.4)/(1.5)</td>
</tr>
<tr>
<td>NLS/MNL</td>
<td>197</td>
<td>1/0</td>
<td>2/0</td>
<td>8/0</td>
</tr>
<tr>
<td>(percentages)</td>
<td></td>
<td>(0.5)/(0)</td>
<td>(1.0)/(0)</td>
<td>(4.1)/(0)</td>
</tr>
</tbody>
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One day after the Cy5-labeled, biotinylated 900 bp GFP expression cassette complexed with streptavidin NLS/NL or streptavidin NLS/MNL was microinjected into the cytoplasm of HeLa cells, the number of cells (without nuclear dextran) that contained GFP expression and/or nuclear DNA were determined. For GFP+/DNA+ cells, weak and strong refer to the strength of both GFP and nuclear DNA signal which was correlated in these cells. For GFP+/DNA− cells, weak and strong refer to the strength of the GFP signal. For GFP−/DNA+ cells, weak and strong refer to the strength of the DNA nuclear signal.
harvested after about 4 hours (Fig. 2). Complexes containing 70 bp - 510 bp DNAs all accumulated relatively efficiently with the majority of the microinjected nuclei containing a strong signal (Fig. 2B,D,G,I). Complexes with ~1 kb DNA still accumulated, but only approximately 10% of the nuclei had substantial staining to the degree shown in Fig. 2K. (see Table 1 for quantitation of a 900 bp DNA fragment). Complexes with 2 or 3 kb DNA yielded weakly-positive signals in only a few percentage of nuclei (data not shown). The DNAs smaller than 310 bp entered nuclei relatively efficiently without any streptavidin-NLS/NL (compare Fig. 2A,C,E,F). A 900 bp DNA fragment did not enter the nucleus as efficiently when complexed with non-functional streptavidin-NLS/MNL (Fig. 6F and Table 1).

Fig. 5. Effect of DNA size on its nuclear accumulation in the presence of RRL in digitonin-permeabilized cells at 37°C and enhancement by binding streptavidin-NLS/NL. 70 bp (A), 70 bp + streptavidin-NLS/NL (B), 110 bp (C), 110 bp + streptavidin-NLS/NL (D), 200 bp (E) 310 bp (F), 310 bp + streptavidin-NLS/NL (G), 510 bp (H), 510 bp + streptavidin-NLS/NL (I), 1 kb (J), 1 kb + streptavidin-NLS/NL (K), 2 kb (L), 2 kb + streptavidin-NLS/NL (M), 3 kb (N) and 3 kb + streptavidin-NLS/NL (O). 200-300ng DNA labeled with one Texas Red or Cy5 fluorophore and one biotin at each end (A-D) or at one end (E-O). Representative examples are displayed. The averages of the mean pixel values in the center of 7-31 nuclei were measured. The values for streptavidin-NLS/NL samples were divided by values without streptavidin-NLS/NL to obtain a ‘+ to –’ ratio (P).

NLS/NL enhancement of GFP expression after microinjection
To better understand the relationship between DNA nuclear transport and gene expression, a 900 bp GFP expression cassette, which was labeled with a biotin on one end and a Cy5 fluorophore on the other end, was complexed with streptavidin-NLS/NL or streptavidin-NLS/MNL. These complexes were microinjected into the cytoplasm of HeLa cells and harvested after 18-24 hours. Only cells with dextran-negative nuclei were analyzed (to exclude cells in which the injected material entered nuclei from either microinjection or cell division). Complexes with functional streptavidin-NLS/NL expressed GFP more efficiently than complexes with non-functional streptavidin-NLS/MNL (Fig. 6 and Table 1). The extent of GFP expression appeared to correlate with the amount of nuclear DNA when the cell expressed at all. However, there were also non-expressing cells with DNA accumulated in the nucleus (Table 1). None of the cells injected with the streptavidin-NLS/MNL complex had strong GFP or DNA staining. The 900 bp GFP DNA without any streptavidin also expressed poorly after cytoplasmic microinjection (data not shown). Initial studies suggest that GFP expression was not enhanced when streptavidin-NLS/NL was complexed with a two kb DNA fragment that contained a GFP expression cassette (data not shown). When the nucleus was compromised during the microinjection experiment (when dextran fluorescence was observed in the nucleus), the 900 bp DNA bound to either streptavidin-NLS/NL or streptavidin-NLS/MNL expressed similarly (18.2% for NLS/NL and 17.7% for NLS/MNL).

The decreased DNA signal in Fig. 6F as compared to 6C may be due to increased degradation of DNA that remains in the cytoplasm as compared to that in the nucleus. Microscopic examination of the microinjected cells at 15 minute, 4 hours, and overnight indicated that there was a several-fold decrease in the cytoplasmic signal from 15 minute to 4 hours, but only a slight decrease thereafter.

DISCUSSION
The size of the linear DNA fragment had a substantial effect on its pattern of nuclear accumulation. DNA less than or equal to 200 bp distributed diffusely throughout nuclei in both digitonin-treated (Fig. 1) and living, microinjected cells (Fig. 2). The ability of DNA less than 200 bp in length to enter the nuclei of digitonin-treated cells at 4°C and without ‘energy’ (Fig. 3A-C) suggests that DNA of this size diffuses passively
mechanism by which these and other proteins translocate these proteins with the nuclear pore have been identified, the nuclear transport of fragments of karyopherin

Recent work shows that the nuclear pore complex (NPC) facilitates the transport of naked DNA into the nucleus. This would put the upper limit for passive nuclear entry of DNA between 132 kDa (200 bp) and 204 kDa (310 bp), which is larger than the upper molecular weight limit for the passive nuclear entry of globular proteins. This may be because naked, linear DNA does not form a globular particle and has a persistence length of ~50 nm (Cantor and Schimmel, 1980). 200 bp DNA, which has a length of about 70 nm and a diameter of about 2 nm, must pass through the nuclear pore peripheral channels that are 9-10 nm in diameter and 100 nm in length (Bustamante et al., 1994). Perhaps if the DNA is too much larger than its persistence length, efficient diffusion through the long skinny channel is inhibited.

DNAs between 310 bp and 1.5 kb in size exhibited less nuclear haze and more punctate staining in the digitonin-treated cells than did DNAs less than 310 bp (Fig. 1). Minimal punctate nuclear staining was observed with the 2 or 3 kb DNA. Thus the upper limit for the effective transport of naked DNA in digitonin-treated cells is approximately 1.5 kb. The cellular basis for this upper limit is not clear given that the DNA in digitonin-treated cells is approximately 1.5 kb. The overall level of uptake was more marked for DNA less than 1.5 kb in length. Since most of the streptavidin complexes contained 2 DNA molecules (Fig. 4), this size limit may be different for single DNA molecules.

The NLS/NL peptide was chosen for these studies because it was more effective than the shorter SV40 T ag NLS in enabling nuclear transport of IgM, a large protein (Jans and Hubner, 1996; Yoneda et al., 1992). The importance of the phosphorylation sites within the NLS/NL peptide for DNA nuclear transport in living cells remains to be determined. Its reduced positive charge (as compared to the +5 charge of the minimal SV40 T ag NLS) could also reduce masking from electrostatic interactions with negatively-charged DNA (Henkel et al., 1992).

Fluorescently-labeled DNA is useful for determining the relative amounts of DNA that physically enter the nucleus. The fluorescent tag should preferably be covalently linked to the DNA since even strong non-covalent DNA binders such as TOTO (Molecular Probes) can move from the exogenous DNA to endogenous chromosomal DNA (J. Wolff et al., unpublished results). Nonetheless, covalently-attached fluorescent tags can also be problematic if DNA degradation occurs. DNA degradation is not an issue in the digitonin-

Fig. 6. An NLS can enhance the transport and expression of DNA. 660 ng/µl 1 – 1 µ g/µl of a biotinylated and Cy5 labeled 900 bp GFP expression vector that was bound to either a functional streptavidin-NLS/NL or a mutant (streptavidin-NLS/MNL) was cytoplasmically microinjected into HeLa cells and harvested after 18-24 hours. DNA + streptavidin-NLS/NL, DAPI channel (A), DNA + streptavidin-NLS/ NL, FITC channel (B), DNA + streptavidin-NLS/NL, Cy5 channel (C), DNA + streptavidin-NLS/MNL, DAPI channel (D), DNA + streptavidin-NLS/MNL, FITC channel (E), DNA + streptavidin-NLS/MNL, Cy5 channel (F).

The signal in E is the same as background in cells without GFP. The best examples are displayed.

The coupling of the karyophilic protein, streptavidin-NLS/NL, to DNAs greater than 110 bp substantially increased their DNA transport in the presence of RRL (Fig. 5). This result is consistent with our previous study (that used the CPI alkylating reagent to covalently attach many NLSs to DNA) and indicates that RRLs inhibitory effect can be overcome by an NLS. In both methods, there is a dose-response relationship between the number of NLSs and the efficiency of nuclear transport. For covalent modification using CPI, the DNA needed to contain at least 3-4 NLSs/100 bp for efficient nuclear entry (Sebestyén et al., 1998), while streptavidin containing 8 NLSs per tetramer transported DNA more efficiently than did streptavidin containing 6 NLSs per tetramer (data not shown). A possible structural correlate to this threshold is the dense docking sites on the cytoplasmic fibrils that extend out from the nuclear pore complex (Feldherr et al., 1984; Richardson et al., 1988).

Linkage of DNA to streptavidin-NLS/NL via biotin increased the nuclear transport of fluorescently-labeled DNA in living cells after microinjection (Figs 2, 6). The overall level of uptake was more marked for DNA less than 1.5 kb in length. Since most of the streptavidin complexes contained 2 DNA molecules (Fig. 4), this size limit may be different for single DNA molecules.

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through the pore is unknown (Mattaj and Englmeier, 1998). Nuclear rimming was observed for DNAs greater than or equal to 310 bp (Figs 1, 3). Perhaps the nuclear translocation of DNA greater than ~1.5 kb is hindered. Studies are in progress to determine whether such DNA is sequestered at the nuclear pores or other peri-nuclear structures.

As the size of DNA increases above 200 bp, the nuclear transport of DNA in microinjected cells diverges from that in digitonin-treated cells (compare Fig. 1 to Fig. 2). Very little punctate staining was observed in cells microinjected with uncomplexed 510 bp or 1 kb DNA. The addition of RRL to the digitonin-permeabilized cells blocked the punctate nuclear staining generated by the 510 bp or 1 kb DNA (Fig. 5), thus paralleling the results in microinjected cells. RRL also altered the intranuclear localization of the 70 and 110 bp DNAs (compare Figs 1 and 5). Studies are in progress to identify the factors in RRL that affect the nuclear transport of DNA in digitonin-permeabilized cells.

The NLS enhanced DNA transport

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permeabilized cell system with or without RRL (Hagstrom et al., 1997) but may be problematic for microinjection. The present study used DNA which contained a fluorescent tag and an NLS at opposite ends. If degradation had occurred in the living cells, then the fluorescent tag would have separated from the NLS and the nuclear fluorescent signal would not be dependent upon the presence of an NLS. The fact that fluorescently-labeled DNA alone or linked to streptavidin containing a mutant-NLS control peptide entered the nucleus less efficiently argues against the possibility that the enhanced nuclear transport of the DNA and streptavidin-NLS/NL complexes is due to DNA degradation. The substantially decreased nuclear signal in cells microinjected with complexes of 2 kb DNA and streptavidin-NLS/NL complexes also argues against DNA degradation (data not shown). In addition, streptavidin-NLS/NL complexed with DNA containing fluoroscein and biotin at one end and Texas Red at the other end was also used for microinjections. The fluoroscein and Texas Red nuclear signals were congruent (data not shown). Even though cytoplasmic degradation of DNA may occur, the nuclear signal observed with streptavidin-NLS/NL bound to biotin DNA is not a result of degradation.

The effect of NLS conjugation on DNA expression is another measure of DNA nuclear transport. Given that the effect of streptavidin-NLS/NL was greater for DNA of a smaller size, a minimal GFP expression cassette was designed to be 900 bp. If the minimal cassette alone or conjugated with a non-functional NLS was microinjected cytoplasmically, approximately 1% of the HeLa cells whose nuclei were intact expressed GFP. This low expression is mainly due to the abbreviated CMV promoter. Linkage of this biotinylated 900 bp GFP expression cassette to the functional NLS (streptavidin-NLS/NL) resulted in GFP expression in ~10% of the cells whose nuclei were intact (Table 1). Strong GFP expression (i.e. Fig. 6B) was observed in 4% of the cells injected with complexes containing the functional NLS but never in cells injected with complexes containing mutant NLS. These results are consistent with the increased nuclear entry of fluorescent DNA conjugated to the streptavidin-NLS/NL (Figs 2, 5, 6). Several cells microinjected with DNA conjugated to the functional NLS had strong nuclear DNA fluorescence but no GFP expression (Table 1). Given that DNA degradation is an unlikely explanation (see above paragraph), the delivered DNA may not be located within a nuclear compartment capable for expression or the cell is not in a proper state for expression. These effects may be heightened by the weakness of the expression vector.

In conclusion, this study demonstrates that the covalent modification of a DNA (by the attachment of the biotin ligand) can enable its increased expression in mammalian cells. A recent report describes the ability for an NLS to increase expression but it did not ascertain the NLSs effect on actual DNA transport into the nucleus by physical methods (Zanta et al., 1999). Our report is the first to correlate the effect of an NLS on both DNAs nuclear transport and expression in the same cell.

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REFERENCES


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