Actin Cytoskeleton as the Principal Determinant of Size-dependent DNA Mobility in Cytoplasm

A NEW BARRIER FOR NON-VIRAL GENE DELIVERY*

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The cytosol of mammalian cells is a crowded environment containing soluble proteins and a network of cytoskeletal filaments. Gene delivery by synthetic vectors involves the endocytosis of DNA-polycation complexes, escape from endosomes, and diffusion of non-complexed DNA through the cytosol to reach the nucleus. We found previously that the translational diffusion of large DNAs (>250 bp) in cytoplasm was greatly slowed compared with that of smaller DNAs (Lukacs, G. L., Haggie, P., Seksek, O., Lechardeur, D., Freedman, N., and Verkman, A. S. (2000) J. Biol. Chem. 275, 1625–1629). To determine the mechanisms responsible for size-dependent DNA diffusion, we used fluorescence correlation spectroscopy to measure the diffusion of single fluorophore-labeled DNAs in crowded solutions, cytosol extracts, actin network, and living cells. DNA diffusion (D) in solutions made crowded with Ficoll-70 (up to 40 weight percentage) or soluble cytosol extracts (up to 100 mg/ml) relative to diffusion of the same sized DNAs in saline (D/Dcyto) was approximately independent of DNA size (20–4500 bp), quite different from the strong reduction in D/Dcyto in the cytoplasm of living cells. However, the reduced D/Dcyto with increasing DNA size was closely reproduced in solutions containing cross-linked actin filaments assembled with gelsolin, whereas soluble macromolecules of the same size and concentration did not reduce D/Dcyto. In intact cells microinjected with fluorescent DNAs and studied by fluorescence correlation spectroscopy or photobleaching methods, D/Dcyto was reduced by 5–150-fold (20–6000 bp); however, the size-dependent reduction in D/Dcyto was abolished after actin cytoskeleton disruption. Our results identify the actin cytoskeleton as a major barrier restricting cytoplasmic transport of non-complexed DNA in non-viral gene transfer.

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Gene delivery by non-viral vectors is a complex and inefficient process that involves cellular internalization by endocytosis, escape from endosomes, DNA-polycation dissociation, and diffusion of non-complexed DNA in the cytoplasm to reach the nucleus (1–3). DNA diffusion in the cytoplasm and nuclear import are believed to be rate-limiting determinants of transgene delivery, extending the time that non-complexed DNA is exposed to cytosolic DNases. Remarkably, <0.1% of DNA microinjected into the cytosol is ultimately expressed (4, 5). We measured previously the mobility of differently sized DNAs in cytoplasm by photobleaching cells after microinjection with fluorescently labeled DNAs (6). The principal finding was that DNA diffusion in cytoplasm (Dcyto) was modestly slowed compared with that in saline (Dcyto/Ds ~ 0.2) for small DNA fragments of <250 bp but was greatly reduced (Dcyto/Ds < 0.05) for larger DNAs of >250 bp such as plasmid-sized DNAs. In contrast, the diffusion of dextrans and Ficolls, which are considered to be non-interacting macromolecules, was only mildly dependent on their size up to 500–1000 kDa (7), which is equivalent in molecular mass to a 750–1500-bp DNA fragment. Identification of the mechanism of reduced mobility of large DNAs in cytoplasm has important consequences regarding intrinsic limitations of non-viral gene delivery and in developing strategies to improve its efficacy.

Several factors can in principle reduce the diffusion of a solute in cytoplasm versus saline, including fluid phase viscosity, binding, and crowding by mobile and immobile macromolecules (8). Systematic analysis of the diffusion of a small fluorescein-like molecule in cytoplasm indicated that reduced diffusion in cytoplasm (Dcyto/D0 ~ 0.25) resulted mainly from macromolecular crowding by the relatively high concentration of proteins in cytoplasm of ~100–150 mg/ml (reviewed in Refs. 9 and 10). Fluid phase viscosity, defined as the effective viscosity sensed by a small molecule that does not undergo binding or other macromolecular interactions, has little influence on cytoplasmic diffusion (11, 12). Binding can greatly reduce apparent diffusion, as was found for some enzymes that assemble into macromolecular complexes (13). As a densely charged polyanion, DNA binding to cytoplasmic components could be an important factor in reducing its diffusion in cytoplasm as was found in the nucleus, where DNA is nearly immobile probably because of binding to positively charged histones (6, 14). DNA diffusion in heterogeneous polydisperse media is further complicated by conformation effects resulting in persistence length and other size-dependent phenomena. Although theoretical descriptions exist in the polymer field for DNA conformational mechanics and electric field-dependent convection in gels (15), the basis of the strong size dependence of DNA diffusion in cytoplasm is not clear on theoretical grounds.

The purpose of this investigation was to establish the mechanism of the reduced diffusion of DNA in cytoplasm with increasing DNA size. Several possibilities were considered, including DNA binding to cytosolic components, molecular crowding by mobile obstacles, and restricted diffusion due to fixed structures. We used fluorescence correlation spectroscopy of DNAs labeled with a single fluorescent probe to measure...
DNA mobility at the single molecule level in crowded solutions and living cells, as well as photobleaching recovery of multiply labeled DNAs in cells. Based on a series of in vitro and cell measurements, we conclude that macromolecular crowding by fixed obstacles, mainly the actin cytoskeleton, is the principal determinant of the size-dependent slowing of DNA diffusion in cytoplasm. Our results have important implications regarding limitations in gene transfer using non-viral vectors.

MATERIALS AND METHODS

Fluorescently Labeled DNAs—Linear double-stranded DNAs of different sizes containing a single rhodamine green molecule were generated by PCR using pCNA 3.1 as the template. DNAs in sizes of 100, 250, 500, 1000, 2000, and 4500 bp were prepared by using a common 5'-end rhodamine green-labeled primer (5'-ATCGTATTACCGATGTTACGCGTTTGG-3') and specific 3'-end primers for each size. The amplified single fluorophore-labeled DNAs were purified by ethanol precipitation and gel filtration and concentrated on Centricron 30 or 100 filters. Rhodamine green-labeled dextran were prepared as described previously (16). The 20-mer (5'-TATTATCTACTGACGTATTACGCGTTT GG-3') and the 50-mer (5'-AAATAGTTGCGACCTGCACGCGTTAATTAATATAAACATGATTA TAT-3') primers containing a 5'-end rhodamine green label were synthesized by Invitrogen along with unlabeled complementary sequences for annealing to give double-stranded DNA. For photobleaching experiments, DNAs were fluorescently labeled with the bis-intercalating dye YOYO-3 (Molecular Probes). One YOYO-3 molecule per 100 bp DNA was found to be optimal for photobleaching studies.

In Vitro Sample Preparation—Labeled DNAs and dextran (10 kDa and 500 kDa) were dissolved at concentrations of 1–100 nm in PBS containing 0.1% bovine serum albumin. In some experiments, the solutions contained Ficoll-70 (0–40 weight percentage), cytosol (0–100 mg/ml), actin (0–8 mg/ml), or actin/gelsolin (actin 8 mg/ml; gelsolin 0.04 mg/ml). Cytosol was prepared from mouse liver. After perfusion, livers were homogenized in buffer containing 320 m_M sucrose, 2 mM MgCl2, 1 mM diithiothreitol, 20 mM Heps (pH 7.4), and several protease inhibitors (2 mg/ml peptatin, leupeptin, aprotinin, and 50 mM Pefabloc®). The homogenate was centrifuged at 10000 × g for 30 min, and the supernatant was centrifuged at 100000 × g for 1 h to separate membranes from the cytosol. All steps were done at 4 °C. Protein concentration was measured by a Coomassie blue assay (Bio-Rad) using bovine serum albumin as a standard. Cytosol was frozen and stored at −80 °C in the presence of 2 mM EDTA until use (17). Cytosol at 100 mg/ml was obtained from 60 mg/ml cytosol by evaporation. For actin-containing solutions, lyophilized rabbit skeletal muscle G-actin protein (Cytoskeleton, Denver, CO) (in stock buffer) was mixed with lyophilized human plasma gelsolin (Cytoskeleton, Denver, CO) to 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 50 mM NaCl at a molar ratio of 400:1. Polymerization was initiated by adding KCl to 50 mM, MgCl2 to 2 mM, and ATP to 1 mM using a small volume of a concentrated stock solution, and the sample was mixed with a micropipette and allowed to stand for 20 min. For fluorescence correlation spectroscopy (FCS) measurements, 5 μl of solutions were sandwiched between two glass coverslips and transferred to the microscope stage.

Fluorescence Correlation Spectroscopy—FCS measurements were performed on a Nikon TE-200 inverted epifluorescence microscope equipped with a 100X objective lens (Nikon, Japan). Filter combinations were 488 nm, 20 m_Watt Millennia; Coherent Inc.), neutral density filter wheel, 100× oil objective (Nikon S Fluor, NA 1.3), 510-nm dichroic mirror, and 535 ± 25 nm bandpass emission filter (Chroma). Fluorescence was collected by a 100-μm-diameter fiberoptic patch cord mounted on a three-axis micropositioner and detected by an avalanche photodiode (<50 counts per minute of dark noise; PerkinElmer Life Sciences). Photon counts were correlated online using an ALV-5000 correlator card. Data recording times were 30–1500 s. Autocorrelation functions, G(τ), were binned and displayed on a quasi-logarithmic time scale. Correlation times, τc, were computed from G(τ) as shown in Equation 1.

\[
G(\tau) = N^{-1} \cdot [1 + (\tau/\tau_c)^{1/2}]^{-1} \cdot [1 + (\tau/\tau_c)^{1/2}]^{-0.5}
\]

(Eq. 1)

\( N \) is fluorophore number density in the detection volume, \( \tau \) is time, and \( \tau_c \) is the characteristic diffusion time for an ellipsoidal excitation volume (\( \tau = 2\pi \eta V / D \)) and eqatorial (\( \eta \)) radii of the focal volume. Diffusion coefficients were determined from fitted \( \tau_c \) using a rhodamine green solution as standard (diffusion coefficient of 2.8 × 10⁻⁶ cm²/s; Ref. 18). Generally, data from 10–40 individual G(τ) curves were averaged. Measurements were done at 23 °C in a temperature-controlled darkroom on a vibration isolation table.

Photobleaching Experiments—Spot photobleaching measurements were performed on an apparatus consisting of an argon ion laser (488 nm; Coherent Inc.), a high contrast acousto-optic modulator, and an inverted epifluorescence microscope (Diaphot, Nikon) equipped with an objective lens (20× dry, numerical aperture 0.75, or 60× oil immersion, numerical aperture 1.4; Nikon) and a 510-nm dichroic mirror (13). Emitted fluorescence was filtered by a long-pass filter (530 ± 15 nm) and cut-on (>515 nm) filters and detected by a gated photomultiplier and 14-bit analog-to-digital converter. For most experiments, the laser beam power was set to 50–100 milliwatts, and the attenuation ratio (the ratio of bleach to probe beam intensity) was ~5000 to give <30% bleach. Data from 10–15 individual recovery curves were averaged, each obtained from a different spot. Diffusion coefficients (in cm²/s) were determined from τc values derived by non-linear regression (13) using fluorescent solution as standard (diffusion coefficient of 2.8 × 10⁻⁶ cm²/s; Ref. 18).

Cell Culture and Microinjection—HeLa cells were cultured in modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO₂ humidified atmosphere. Solutions for microinjection consisted of calcium-free PBS containing 0.5 μg/ml rhodamine green dye, 0.5 μM latrunculin B (DNAmobile), and 5242 microinjector. Glass needles were drawn from thin-walled filament capillaries (FHC, Brunswick, ME) with a vertical needle puller (Kopf, Tujunga, CA). Cells grown on 18-mm-diameter round glass coverslips were microinjected (generally ~200 cells injected on each coverslip at an injection pressure of 120–200 kPa) by a Leitz upright fluorescence microscope equipped with a coaxial-confocal attachment and a 100× objective.

RESULTS

Characterization of DNA Fragments Containing a Single Fluorescent Label—Linear double-stranded DNAs containing a single fluorescent label were prepared by PCR using a 5'-rhodamine green labeled primer and different 3'-end primers to amplify DNAs of specified sizes (Fig. 1A). The size, integrity, and purity of the linear DNAs after purification were confirmed by agarose gel electrophoresis with ethidium bromide staining and by rhodamine green fluorescence (Fig. 1B). The rhodamine green chromophore was used as a fluorescent probe for FCS measurements because of its photostability and absence of significant photophysical phenomena (such as triplet state relaxation and flicker) under the conditions of our experiments and its suitable excitation spectrum for diode laser excitation.

Diffusion coefficients of the rhodamine green-labeled DNAs were measured by FCS of representative normalized autocorrelation curves, G(τ)/G(0), as shown in Fig. 1C. Data for all DNAs fitted well to the model in Equation 1 containing a single correlation time, τc (residence time in the illuminated region), indicating simple (non-anomalous) diffusion. Correlation times increased with DNA molecular size, with τc increasing from 0.7 ± 0.1 ms (mean ± S.D.) for 20-bp DNA to 28 ± 5 ms for 4.5-kb DNA. Control experiments were done for each DNA to confirm the validity of τc values, including showing independence of τc on 4-fold changes in illumination intensity and concentration and the absence of photobleaching over the duration
of data acquisition. Fig. 1D summarizes deduced translational diffusion coefficients as a function of DNA molecular size on a log-log plot with an empirical linear fit, $D_0 = 6.5 \times 10^{-13}$ cm$^2$/s (base pair)$^{-0.08}$, which is $r^2 = 0.999$. The fitted slope is in agreement with the slope of 0.70 predicted from the theory of Yamakawa and Fujii (19) for helical worm-like molecular chains without excluded volume. An alternative model (20) also fitted the data well; the dashed curve labeled model (Fig. 1D) was computed from the equation of Tirado and Garcia de la Torre, $D_0 = (k_BT/3\pi\eta L)ln(p + v)$, where $v = 0.312 + 0.565/p - 0.1/p^2$ is a correction factor for end effect, $p = L/d$ is the ratio of DNA length to diameter, $k_BT$ is the product of Boltzmann constant and temperature, and $\eta$ is medium viscosity. The poorly fitting dashed line labeled sphere (Fig. 1D) is shown for comparison and was computed for spherical molecules of equivalent molecular weight to the DNAs.

**Macromolecular Crowding Effects on DNA Diffusion**—DNA diffusion was measured in solutions made crowded with various soluble (mobile) and fixed (immobile) macromolecules to establish the determinants of size-dependent DNA diffusion. Diffusion coefficients for DNAs were measured first in saline solutions made crowded with the soluble “crowding agent” Ficoll-70 (0–40 weight percentage). As in many prior studies of solutions made crowded with the soluble “crowding agent” Ficoll-70 (0–40 weight percentage), Ficoll-70 was chosen as a macromolecular crowding agent (9, 21). Diffusion coefficients for DNAs were measured in solutions made crowded with various molecular weight to the DNAs.

**Actin Cytoskeleton Reduces DNA Diffusion**—DNA diffusion was measured in solutions made crowded with various soluble proteins and macromolecules, the cell cytoplasm contains a network of cytoskeletal filaments, among which microfilamentous F-actin appears to be the most prominent (24). To test whether an actin network could account for the size-dependent DNA diffusion, an actin mesh was generated in vitro using purified actin at concentrations found in cells (5–12.5 mg/ml) (25, 26) alone or together with the actin-binding protein gelsolin. $D/D_0$ was mildly reduced with DNA size at 1 mg/ml actin and substantially reduced at 8 mg/ml of actin alone or together with gelsolin (Fig. 3). For each actin concentration, DNA diffusion coefficients were at least 10–100-fold higher than the diffusion coefficients for polymerized actin filaments ($<10^{-11}$ cm$^2$/s; Ref. 27), making the actin mesh effectively immobile on the time scale of DNA diffusion.

Fig. 1. Syntesis and characterization of fluorescently labeled DNAs. A, schematic of the PCR-based DNA synthesis method showing incorporation of a single 5'-rhodamine green-labeled (RhodGreen) primer into the amplified DNA fragment. B, agarose gel electrophoresis of PCR-amplified DNA fragments. The single rhodamine green (Rhod green) fluorophore at the 5'-end was visualized by fluorescence using a Typhoon 8600 apparatus (Amersham Biosciences). C, normalized autocorrelation data, $G(t)/G(0)$, and fitted curves (smooth curves) of rhodamine green-labeled linear double-stranded DNA fragments (5–50 nM) in saline containing 0.1% albumin at 23 °C with a 50-s acquisition time. $D_0$, diffusion coefficients as a function of DNA size computed from data in panel C shown on a log-log plot (mean ± S.D., n = 15–40 measurements). Theoretical curves of size-dependent DNA diffusion plotted according to the equations of Tirado and Garcia de la Torre (model) or for an equivalently sized sphere (see “Results”).

Fig. 2. Influence of macromolecular crowding by mobile obstacles on DNA mobility. A, diffusion coefficients of DNA fragments of indicated size as a function of concentration of the crowding agent Ficoll-70 (mean ± S.D., n = 10–30 measurements). B, ratio of the relative DNA diffusion in Ficoll-70 to that in saline, $D/D_0$, as a function of DNA size and Ficoll-70 concentration. C, representative normalized autocorrelation data, $G(t)/G(0)$, and fitted curves (smooth curves) of rhodamine green-labeled, linear, double-stranded, 100-bp and 1-kb DNA fragments in soluble cytosol extract at 8 and 100 mg/ml protein. $D_0$, ratio of the relative DNA diffusion in cytosol extracts to that in saline as a function of DNA size (mean ± S.D., 10–20 measurements).
diffusion. For comparison, the diffusion of rhodamine green-labeled dextrans (10 and 500 kDa) in 1 and 8 mg/ml actin networks is shown. Both dextrans (equivalent in molecular weight to 15-bp and 750-bp DNAs) diffused almost freely in the actin network, indicating little influence of the actin network on the diffusion of globular non-interacting macromolecules with 5–30 nm gyration radii (23).

**DNA Diffusion in the Cytoplasm of Living Cells—FCS measurements in cells were conducted under the same conditions as in the in vitro experiments using a 100× objective lens, low laser illumination intensity (<0.2 milliwatts), and room temperature (to minimize nucleic-acid-induced DNA degradation) (17, 28). HeLa cells were microinjected with rhodamine green-labeled DNAs and kept at room temperature for 20 min prior to measurements. The laser spot was focused in an area of cytoplasm not directly adjacent to the nucleus or the cell membrane. In initial studies, FCS data were stable at 15–30 min after microinjection, but changes were seen at longer times or after 37°C incubation, which may be related to nucleic-acid-dependent generation of smaller degradation fragments. Representative FCS curves for 3-kb DNA are shown in Fig. 4A, and deduced \( D/D_0 \) values are summarized in Fig. 4B. For the larger DNAs (250–4500 bp), the fitting of correlation functions required a two-component model. The fit shows a fast correlation time component, \( \tau_{ct} = 5–20 \) ms, which was independent of DNA size, and a slower correlation time, \( \tau_{ct} \), that depended on DNA size and cell maneuvers. The rapid correlation time component may be related to a small amount of degraded DNA, rhodamine green-related photophysics, and/or intramolecular DNA motion/local confinement effects in a heterogeneous environment. Other studies reported similar two-component models for the analysis of fluorescent macromolecule diffusion in cells (29–31).

As found previously in photobleaching measurements of fluorescein isothiocyanate-labeled DNAs (6), diffusion of DNA with a single rhodamine green as measured by FCS was remarkably reduced with increasing DNA size. To test the involvement of the actin cytoskeleton in the reduced DNA diffusion, FCS measurements were done in cells after actin skeletal disruption with cytochalasin D. Fig. 4B shows that actin skeletal disruption largely eliminated the size-dependent reduction in DNA diffusion, providing in vivo support for the conclusion from solution studies that the actin mesh is an important determinant of DNA diffusion. Fig. 4B also shows a curve labeled predicted that was computed from the product of the in vitro \( D/D_0 \) values in the cytosol (100 mg/ml) and the actin/gelsolin mesh. The “predicted” curve is in good agreement with \( D/D_0 \) measured experimentally in cells, consistent with the view of the cytoplasm as a crowded soluble phase surrounded by an entangled filament network.

Because FCS is based on the analysis of fluorescence intensity fluctuations produced by the diffusion of molecules into and out of a defined excitation volume, the identification and quantification of an immobile population of molecules is not possible. Because of this limitation as well as the complexities in the interpretation of FCS data in living cells (see “Discussion”), photobleaching experiments were done to confirm the conclusion that the actin cytoskeleton is the main determinant of the reduced diffusion of DNA in cytoplasm. For these studies we used a novel labeling strategy to generate brightly fluorescent DNA fragments. DNAs (500 bp and 6000 bp) were labeled with the dimeric cyanine dye YOYO-3, which binds tightly to denatured DNA (32). DNAs (500 bp and 6000 bp) were labeled with the dimeric cyanine dye YOYO-3, which binds tightly to denatured DNA (32). DNAs (500 bp and 6000 bp) were labeled with the dimeric cyanine dye YOYO-3, which binds tightly to denatured DNA (32). Because FCS is based on the analysis of fluorescence intensity fluctuations produced by the diffusion of molecules into and out of a defined excitation volume, the identification and quantification of an immobile population of molecules is not possible. Because of this limitation as well as the complexities in the interpretation of FCS data in living cells (see “Discussion”), photobleaching experiments were done to confirm the conclusion that the actin cytoskeleton is the main determinant of the reduced diffusion of DNA in cytoplasm. For these studies we used a novel labeling strategy to generate brightly fluorescent DNA fragments. DNAs (500 bp and 6000 bp) were labeled with the dimeric cyanine dye YOYO-3, which binds tightly to denatured DNA (32).

**Discussion**

The goal of this work was to determine the mechanism of the reduced mobility of non-complexed DNAs in cytoplasm with increasing DNA size. Fluorescence correlation spectroscopy was used to follow DNA diffusion in vitro and in living cells. We found that labeling with a single rhodamine green fluorophore
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was suitable for the investigation of DNA dynamics in complex and in crowded environments using FCS. The PCR-based labeling approach gave a uniform population of fluorescently labeled DNAs in near native form and is suitable for DNA labeling with a variety of fluorescent and other probes. The principal conclusion is that the actin mesh rather than cytoplasmic crowding by diffusible macromolecules provides the rate-limiting barrier for the cellular diffusion of large, non-complexed DNAs.

The mechanism of size-dependent DNA diffusion in cytoplasm was investigated from measurements of DNA diffusion in artificial solutions containing a non-interacting crowding agent (Ficoll-70), concentrated soluble cytosolic extracts, and in vitro skeletal actin network. Diffusion of DNA in solutions made crowded with Ficoll-70 could be reduced considerably compared with diffusion of the same sized DNA in saline. However, the DNA size-dependent reduction in $D/D_0$ could not be reproduced, indicating that molecular crowding by mobile obstacles cannot account for the observed reduction in $D/D_0$ in cells. Reduced $D/D_0$ with increasing DNA size also could not be reproduced in vitro in cytosol at a protein concentration similar to that in cells and in which DNA diffusion was slowed ~5-fold compared with its diffusion in saline. Therefore, crowding effects and DNA interactions with soluble components of cell cytosol cannot account for the reduced $D/D_0$. However, reduced $D/D_0$ with increasing DNA size could be reproduced in vitro in a polymerized actin/gelsolin mesh at a concentration found in the actin mesh is the primary mechanism responsible for the size-dependent reduction in DNA diffusion.

$D/D_0$ in the presence of a combination of mobile obstacles and a polymerized actin/gelsolin mesh is, at first approximation, the product of $D/D_0$ for each mechanism. As such, DNA diffusion in cell cytoplasm was closely modeled as a product of $D/D_0$ measured in vitro in a cytosol extract at 100 mg/ml and a polymerized actin/gelsolin mesh. The mobile obstacles are responsible for a multiplicative factor giving comparable reduction in the diffusion of DNAs of all sizes, whereas the actin mesh is responsible for the size-dependent reduction in DNA diffusion. Our analysis thus provides a quantitative accounting for the observed DNA mobility in living cells with a predictive value in assessing the effects of altered protein concentration and skeletal density on DNA diffusion.

Restricted diffusion of non-complexed DNA in cytoplasm is thought to be a key barrier to gene delivery in vivo, where DNA degradation competes with diffusion (1). The release of non-complexed DNA into the cytoplasm was shown using the T7 polymerase expression system (34). Because the lifetime of cytoplasmic DNA is relatively short (60–90 min) (28), the transport of DNA toward the nucleus should be as fast as possible for efficient transgene expression. The photobleaching studies here indicated that the diffusion of plasmid-sized DNA is very slow, with <20% of DNA being able to diffuse through the cytoplasm. However, disruption of the actin network increased both the mobile DNA fraction as well as its diffusivity. Viruses have evolved efficient DNA packaging and intracellular transport mechanisms to deliver their nucleic acids to the nucleus (35). Because diffusion in the crowded cytoplasm is inefficient given the large size of most capsids, viruses often exploit the cytoskeleton and cellular motor proteins to move through the cell. As shown here for non-viral gene vectors, the actin cytoskeleton also poses a barrier against the inward movement of viruses that enter directly through the plasma membrane (36). To overcome the skeletal barrier, some viruses such as SV40 activate tyrosine kinase-induced signaling cascades that lead to the local dissociation of filamentous actin. Because macromolecule-solutes with a gyration radius ($R_g$) up to 30 nm are freely diffusible in the cytoplasm (7), strategies to increase DNA mobility, such as DNA compaction into spherical 30 nm-particles (37) and active transport of the DNA toward the nucleus, are predicted to enhance the efficiency of transgene delivery (38, 39). In summary, using the complementary fluorescence techniques of FCS and photobleaching, we have established a quantitative mechanism for the reduced translational diffusion of large, non-complexed DNAs in cell cytoplasm and identified the actin cytoskeleton as a new barrier for non-viral gene delivery.

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Fig. 5. Photobleaching recovery measurements of YOYO-3-labeled DNA in solution and in the cytoplasm of microinjected HeLa cells. A, fluorescence recovery curves for YOYO-3 labeled, double-stranded 500-bp and plasmid-sized DNAs in saline at 23 °C. Bleach time was 1–3 ms, solution layer thickness was 5 μm, and a 20× objective lens was used. B, fluorescence recovery data (60× oil immersion objective) in non-treated HeLa cells (top) and after cytochalasin D treatment (bottom). In each case, bleach time was <2% of recovery t_{1/2}.
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