

Intracellular Trafficking of Plasmids during Transfection Is Mediated by Microtubules

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Little is known about how plasmids move through the cytoplasm to the nucleus. It has been suggested that the dense latticework of the cytoskeleton impedes free diffusion of large macromolecules, including DNA. However, since transfections do work, there must be mechanisms by which DNA circumvents cytoplasmic obstacles. One possibility is that plasmids become cargo on cytoskeletal motors, much like viruses do, and move to the nucleus in a directed fashion. Using microinjection and electroporation approaches in the presence of drugs that alter the dynamics and organization of the cytoskeleton, we show that microtubules are involved in plasmid trafficking to the nucleus. Further, by co-injecting inhibitory antibodies, we find that dynein likely facilitates this movement. These results were confirmed using an *in vitro* spin-down assay that demonstrated that plasmids bind to microtubules through adaptor proteins provided by cytoplasmic extracts. Taken together, these results suggest that plasmids, like most viruses, utilize the microtubule network and its associated motor proteins to traffic through the cytoplasm to the nucleus.

Key Words: electroporation, gene therapy, transfection, lipoplex, dynein, cytoskeleton

INTRODUCTION

Gene therapy, specifically nonviral gene therapy, has been greatly limited by the relative inability to achieve high levels of expression. To date, most attempts to improve nonviral gene therapy have focused on overcoming the barriers presented by the cell membrane and nuclear envelope or increasing the ability of the plasmids to escape the endosome in lipid-mediated transfections [1]. However, it has been shown that in liposome-transfected cells, after endosomal escape, a significant amount of plasmid remains free in the cytoplasm and does not reach the nucleus, suggesting that it may be a contributing factor to the observed low expression levels [2,3]. Further, when DNA is delivered to the cytoplasm directly by techniques such as electroporation or microinjection, it must navigate through the cytoplasm to reach the nucleus. It has been shown previously that molecules of DNA larger than 2000 bp are unable to diffuse freely in the cytoplasm and that microinjected plasmids do not diffuse far from the site of injection [4,5]. However, it is known that despite this apparent inability of plasmids to diffuse throughout the cytoplasm, transfections do result in expression. Hence, plasmids must be able to traverse the cytoplasm via a means other than diffusion. Understanding how plasmids move through the cytoplasm is

imperative to understand and enhance transfections, as well as the field of gene therapy as a whole.

One possibility is that plasmids utilize the cell's own machinery for transporting molecules through the cytoplasm: the cytoskeletal network. For instance, when cellular DNA in the nucleus is damaged, p53 translocates to the nucleus via dynein, a retrograde molecular motor that travels on microtubules [6–10]. However, it is not only endogenous proteins that use the cytoskeleton to reach the nucleus. Many viruses also utilize microtubules to reach the nucleus, including cytomegalovirus, human immunodeficiency virus, herpes simplex virus, adenovirus, and parvovirus [11–17]. Alternatively, other viruses and certain bacterial pathogens use actin for intracellular movement [18,19].

Despite the realization that DNA movement to the nucleus is possibly one of the most important barriers to transfection, little work has been done to characterize the mechanisms by which plasmids traverse the dense cytoplasm to reach the nucleus [3]. In the present study, we use electroporation and microinjection to show that plasmids move along the microtubule network and likely use dynein as the molecular motor that facilitates movement toward the nucleus. Biochemical experiments confirm the interaction between plasmids and micro-

tubules in the presence of cell extract, suggesting the need for adaptor proteins.

RESULTS

Taxol Stabilization of Microtubules Enhances Expression of Electroporated Plasmids

To determine whether the cytoskeleton plays a role in the intracellular trafficking of plasmids during transfections, we electroporated adherent A549 cells with luciferase-expressing plasmids in the presence or absence of various cytoskeletal-altering drugs. Since these drugs are known to affect endocytosis, if lipoplex reagents were used for transfections in these studies it would be impossible to discern the drugs' effects on endocytosis from those on cytoplasmic trafficking. By contrast, electroporation induces small, transient pores in the cell membrane thereby allowing entry of DNA into the cytoplasm independent of endocytosis [24], ensuring that the drug effects are due to events occurring in the cytoplasm and not the ability of the plasmids to enter the cytoplasm. Two hours after electroporation of the plasmids, we harvested the cells and determined luciferase expression. We chose the 2-h time point to minimize any additional changes that could occur with prolonged drug treatment of the cells. However, we noted the same results following 24 h incubation in the presence of drugs (data not shown). When we used jasplankinoline, which stabilizes the actin cytoskeleton, or latrunculin B, which disrupts the actin cytoskeleton, there was no change in luciferase expression over vehicle-treated cells, suggesting that the actin cytoskeleton is not involved in transport of the plasmids to the nucleus. Disruption of the microtubule network with nocodazole also did not signifi-

cantly alter luciferase expression levels. By contrast, stabilization of the microtubule network by taxol increased luciferase expression approximately 4.5-fold over baseline (Fig. 1).

To ensure that the observed changes in luciferase expression seen upon incubation of cells with taxol were a result of cytoplasmic plasmid trafficking and not altered transcriptional activity, we electroporated cells with the same luciferase-expressing plasmids and waited 24 h to ensure that the plasmids had reached the nucleus before drug treatment. The cells were then treated with the various drugs for 2 h and luciferase expression was measured. Using this approach, any changes in luciferase expression would be due to drug effects on gene expression, independent of cytoplasmic trafficking. Neither disruption nor stabilization of the actin cytoskeleton affected the ability of the plasmids to express once in the nucleus (Fig. 1B). It has been reported that NF κ B can be activated by translocating to the nucleus in some cell lines when cytoskeletal-altering compounds are used [25,26]. Since there is an NF κ B binding site in the CMV promoter in pCMV-Lux-DTS, to confirm that the drug effects were not at the transcriptional level, we used an NF κ B-responsive, luciferase-expressing plasmid in a set of transfections. Using a strategy similar to that just described, we electroporated pNF κ B-Luc into cells and 24 h later treated the cells with jasplankinoline, latrunculin B, nocodazole, or taxol for 2 or 24 h. Again, no changes in luciferase expression were noted (Fig. 1B). We obtained similar results in both A549 and TC7 cells (not shown). Taken together, these results suggest that stabilization of the microtubule network increases intracellular trafficking of plasmids and subsequent gene expression.

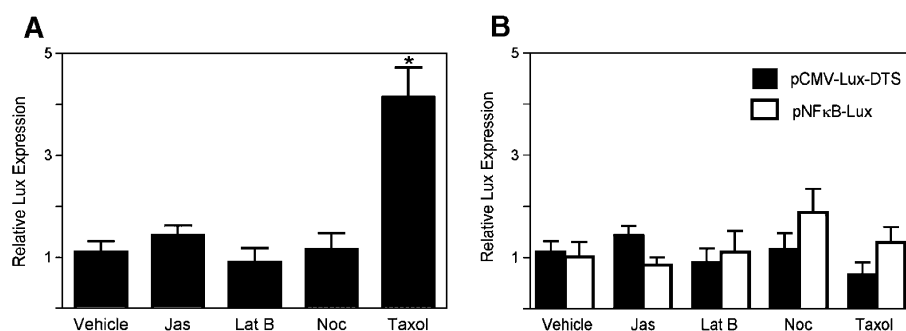


FIG. 1. Stabilization of the microtubule network increases intracellular trafficking and gene expression of plasmids during transfection. (A) A549 cells were electroporated with pCMV-Lux-DTS and immediately treated with vehicle alone (DMSO), jasplankinoline (125 nM), latrunculin B (2.5 μ M), nocodazole (20 μ M), or taxol (10 μ M) for 2 h, after which cells were harvested and luciferase activity was measured. Mean luciferase activities \pm SD (RLU/mg cell protein) were normalized to control transfected cells (no drug or vehicle treatment) and experiments were performed in triplicate and repeated three times. * P < 0.001 by paired Student t test. (B) Plasmid transcription is not greatly altered by drug treatment. A549 cells were electroporated with either pCMV-Lux-DTS or pNF κ B-Luc and 24 h later, the same drugs were added to the cells. Two hours after the addition of drugs, luciferase activities were measured and normalized to control cells as in A.

Disruption of Microtubules Decreases Expression of Microinjected Plasmids

To confirm that microtubules are involved in the trafficking of plasmids to the nucleus, we microinjected green fluorescent protein (GFP)-expressing plasmids into the cytoplasm or nucleus of TC7 cells and treated the cells with either taxol or nocodazole. When the plasmids were injected directly into the nuclei of cells, neither nocodazole nor taxol altered the number of cells expressing GFP or the amount of GFP expressed per cell, confirming that these drugs are not greatly affecting transcription in these cells (Fig. 2A). By contrast, when we injected plasmids cytoplasmically and examined GFP expression 5 h post-injection, we found nocodazole treatment to decrease significantly the ability of the plasmids to express (Fig. 2B). We saw similar results in A549 cells (data not shown). These results again suggest that the microtubule network plays a role in DNA trafficking.

Dynein Inhibition Decreases Expression from Microinjected Plasmids

Most trafficking of cargo along microtubules toward the nucleus uses the major minus-end-directed motor dynein. As such, we sought to determine if inhibition of dynein would also inhibit intracellular plasmid movement. To address this, we co-injected the GFP-expressing plasmids with either anti-dynein or control antibodies and measured GFP expression 5 h later (Fig. 2). When the plasmids and antibodies were co-injected into the nucleus, we noted no change in GFP expression (Fig. 2A). However, when the plasmids were cytoplasmically injected, co-injection with anti-dynein, but not the control antibody, significantly decreased GFP expression levels, suggesting that dynein plays a role in DNA movement (Fig. 2B).

An Intact Microtubule Network Is Required for Efficient Nuclear Localization of Plasmids

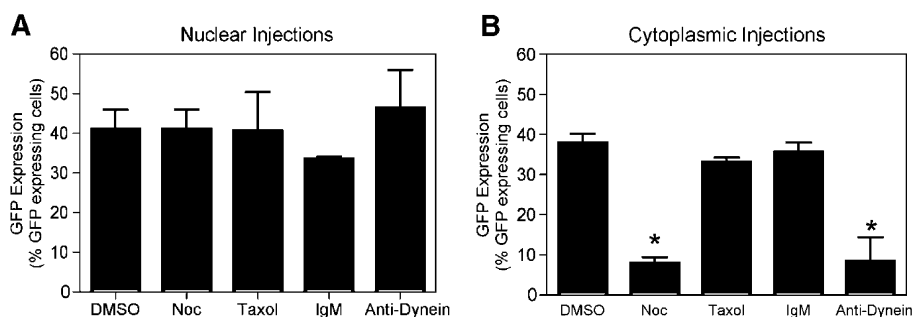
The previous data suggest that plasmids utilize the microtubule network and the retrograde motor dynein for intracellular trafficking to the nucleus. However,

these data are based on the downstream readout of gene expression. To visualize plasmid trafficking directly, we labeled plasmids with fluorescent peptide nucleic acids (PNAs) and cytoplasmically microinjected them into TC7 cells. One hour prior to injection, we treated the cells with either nocodazole to disrupt the microtubule network or vehicle alone (DMSO) and maintained the drugs in the culture medium following microinjection. Immediately following microinjection, the labeled DNA was evenly distributed either throughout the cytoplasm or near the site of injection (not shown). At 5 h post-injection, the majority of the plasmids in cells treated with vehicle alone were localized either within the nucleus or directly around it (Fig. 3A). In fact, we observed strong nuclear localization in most vehicle-treated cells by 3 h. By contrast, in cells treated with nocodazole, plasmids were located diffusely throughout the cell and did not demonstrate any specific pattern of localization at 5 h postinjection (Fig. 3B). These results suggest that the effects of microtubule organization-altering drugs affect plasmid trafficking directly through the cytoplasm.

Plasmids Interact with Microtubules in the Presence of Cellular Proteins

Taken together, these findings suggest that plasmids interact with microtubules via dynein and other proteins. Consequently, we sought to confirm *in vitro* that plasmids could, in fact, interact with microtubules. We used a microtubule spin-down assay, in which plasmid DNA, cell extracts, and stabilized microtubules were allowed to interact and then centrifuged to separate polymerized microtubules and any proteins or DNA interacting with the microtubules from the reaction. After separating the pellet (containing polymerized microtubules) and the supernatant, we performed quantitative PCR to determine the location of the DNA (Fig. 4). When the assays were performed in the absence of microtubules or in the presence of unstabilized tubulin (in the absence of taxol), plasmid was found only in the supernatant, confirming

FIG. 2. Disruption of the microtubule network or inhibition of dynein results in decreased DNA trafficking and gene expression following cytoplasmic microinjection of plasmids. TC7 cells were microinjected with pCMV-GFP-DTS (0.5 mg/ml) into the (A) nucleus or (B) cytoplasm and 5 h later, the percentage of GFP-expressing cells was determined. Cells were incubated with vehicle (DMSO) or nocodazole (20 μ M) immediately following microinjection of plasmid or co-injected with a control IgM (IgM) or the anti-dynein 70.1 antibody (Anti-Dynein) along with pCMV-GFP-DTS. At least 100 cells were injected for each condition and the experiment was repeated three times (mean % expressing cells \pm SD).



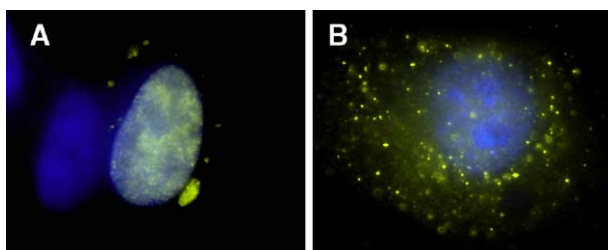


FIG. 3. Disruption of the microtubule network inhibits trafficking of plasmids to the nucleus. A derivative of pCMV-GFP-DTS was fluorescently labeled with Cy3-PNA and the labeled plasmid (0.5 mg/ml) was microinjected into the cytoplasm of TC7 cells that had been pretreated for 1 h with (A) DMSO (vehicle) or (B) nocodazole (20 μ M). Five hours after injection and continued drug treatment, plasmids were visualized in the living cells. Images are representative of over 200 injected cells in three separate experiments.

that it does not pellet on its own. DNA also failed to pellet when incubated with either stabilized microtubules or cytoplasmic extracts alone. However, when we added cell extract to stabilized microtubules, the plasmid was found in the pellet with the microtubules. Further, increasing amounts of DNA pelleted with the microtubules as the amounts of cell extract were increased. When we used a similar spin-down assay to determine whether plasmids could also interact with actin filaments, we detected no interaction under any condition, with or without cell extracts (not shown). Taken together, these results demonstrate that plasmid can interact, most likely through adapter proteins, with microtubules for movement through the cytoplasm.

DISCUSSION

When plasmids enter the cytoplasm by means such as electroporation or microinjection, they need to travel at distances ranging from 1 μ m in small cells to hundreds of micrometers in larger cells. Yet, since diffusion through the dense meshwork of the cytoplasm is not likely, there must be other mechanisms by which plasmids navigate through the cytoplasm [4]. Understanding this intracellular trafficking is imperative to improve the ability of plasmids to reach the nucleus following not only electroporation or direct injection, but all methods of transfection. For instance, it has been shown that lipid-DNA complexes can accumulate in the perinuclear region after endocytosis, but following endosomal escape, the DNA must still travel some distance to enter the nucleus [3,27]. Therefore, regardless of the method by which DNA is introduced into a cell, at some point it must traverse the cytoplasm to reach the nucleus.

In this study, we found that reorganization of the microtubule network greatly affected plasmid trafficking and expression. Stabilization of the microtubule network led to enhanced plasmid expression in electroporated cells, while disassembly of the microtubules decreased expression and trafficking in microinjected cells. Further,

inhibition of dynein, the microtubule-associated retrograde motor, decreased DNA trafficking and subsequent gene expression in microinjected cells. The ability of plasmids to interact with microtubules was confirmed using *in vitro* binding assays. These results demonstrate that plasmids, like various cellular and exogenous proteins and viruses, use the microtubule network for retrograde transport to the nucleus.

It was rather surprising that the effects of drug treatment were not identical in both gene transfer systems. Whereas microtubule disassembly inhibited gene expression following microinjection, it had no effect on expression in electroporated cells, and similarly, while microtubule stabilization increased gene expression in electroporated cells, it did not in injected cells. One possibility for these seemingly disparate results could be due to the initial intracellular distribution of plasmids following the two different delivery methods. When cells are electroporated, DNA enters the cell across the entire plasma membrane and is distributed throughout the cytoplasm, relatively uniformly [28,29]. Consequently, some of the plasmids enter the cell in close proximity to the nucleus, if one assumes that epithelial cells in culture resemble fried eggs. The expression measured in nocodazole-treated cells may be simply a reflection of plasmid that enters the cytoplasm in the immediate vicinity of the nucleus, whereas plasmid on the inner face of the plasma membrane at locations far from the nucleus may indeed fail to traffic in the absence of microtubules in these cells. Microinjection, on the other hand, deposits plasmids at one point far from the nucleus (based on our method of microinjection) and as such would be more affected by the loss of the microtubule network. Similar logic could account for why stabilization of the microtubule network did not increase

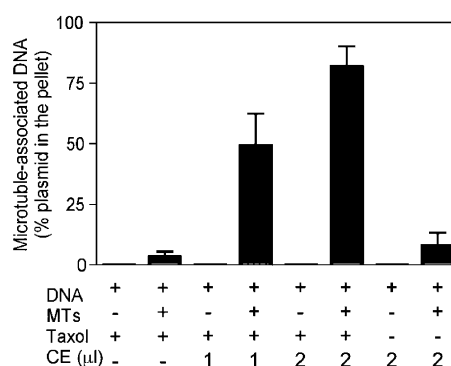


FIG. 4. Quantitative analysis of DNA association with microtubules. Non-polymerized tubulin or taxol-stabilized microtubules were incubated with pCMV-Lux-DTS in the presence or absence of cell extract (10 mg/ml) and subsequently separated over a glycerol cushion by centrifugation. The plasmid content of the pellets (containing polymerized microtubules and associated proteins/DNA) and supernatants was determined by real-time quantitative PCR to determine where the plasmid localized. Mean DNA concentrations from three independent experiments, performed in duplicate, are shown \pm SD.

expression in microinjected cells: with the majority of plasmids concentrated at one place, largely unable to diffuse, access to microtubules is limited. Thus, even with an increased number of stabilized microtubules, the plasmids may be unable to reach and utilize them for increased trafficking.

Verkman and colleagues have shown that microinjected DNA fragments greater than 2000 bp in length show no translational diffusion through the cytoplasm [4]. More recent work from this group has shown that the actin cytoskeleton is the principle structure that limits this passive diffusion through the cytoplasm and that disruption of the actin cytoskeleton by drugs results in an increase in the diffusion of large macromolecules or linear DNA fragments [30]. Similar earlier findings from Popov and Poo support these studies [31]. Although we see no effect on intracellular plasmid trafficking upon stabilization or disassembly of the actin cytoskeleton, this may not be counter to these reports. Indeed, our study has focused not on measuring rates of diffusion through the cytoplasm on small time scales (seconds to minutes), but rather the directed movement of DNA on longer time scales (hours) as measured by gene expression and subcellular localization. Thus, disruption of the actin network may play a significant role in local movement over very short times, but this role may be lessened over time, as microtubules play the predominant role in DNA movement.

Our results strongly suggest that plasmid DNA uses microtubules and dynein to reach the nucleus, but the mechanisms of DNA-dynein complex formation are as yet unknown. Recent work by Mesika *et al.* has shown that the addition of protein nuclear localization signals (NLSs) to plasmids facilitates their transport in the cytoplasm, based on analysis of fluorescently labeled plasmids following microinjection [32]. When plasmids containing binding sites for the p50 subunit of NF κ B were complexed with the protein and microinjected into cells, the plasmids localized to the area around the nuclear envelope and even in the nucleus. Further, this movement appeared to be dependent on dynein, since its inhibition decreased the nuclear localization of the complexes. By contrast, when uncomplexed plasmids were injected into the cells, very little movement was detected. In other studies, Hanz *et al.* demonstrated that importin- α was constitutively bound to dynein in neurons and could form a complex with importin- β and NLS-containing proteins to transport the complex to the nucleus along microtubules [33]. More recent work from this group has reported that the protein vimentin can bind to importin- β through an NLS-independent mechanism and that this complex can interact with dynein and microtubules, through importin- α , for transport through the axon to the cell body, demonstrating that multiprotein complexes can be formed for transport along the microtubule network [34].

We and others have demonstrated that nuclear import of plasmids is sequence-dependent, and we have identified several DNA sequences that mediate DNA nuclear import [35–39]. All of these DNA nuclear targeting sequences (DTSs), including the SV40 DTS, bind to multiple transcription factors, and since transcription factors function in the nucleus and thus contain NLSs, the DTS becomes coated with NLSs provided by these proteins. Our current model is that these NLSs on the DNA-bound transcription factors can then facilitate entry of the DNA-protein complex into the nucleus in an importin- α/β - and RAN-dependent process [37,40–42]. Based on this model, it is also possible that these same transcription factors could serve as adapters between the plasmid, importin- α/β , and dynein, to promote cytoplasmic trafficking of the DNA complexes toward the nuclear envelope. However, all of the plasmids used in this study contain not only the SV40 DTS, but also other eukaryotic control elements such as promoters, introns, and polyadenylation sites, suggesting that perhaps these sequences may act as scaffolds for the adapter proteins. Thus, whether microtubule trafficking of plasmids requires specific sequences, and what these sequences may be, remains to be seen.

MATERIALS AND METHODS

Cell culture and electroporation. TC7 cells, a subline of African green monkey kidney epithelium, and human adenocarcinoma A549 cells (ATCC, No. CCL-185, Rockville, MD, USA) were grown in DMEM supplemented with 10% fetal bovine serum. For electroporations, cells were grown to confluency in six-well dishes and rinsed twice in serum- and antibiotic-free medium. Ten micrograms of plasmids in 1 ml of serum- and antibiotic-free DMEM was added to each well and one 165-mV square-wave electric pulse was applied using a PetriPulser electrode (BTX, San Diego, CA, USA). Immediately following electroporation (within 15 s), the indicated drug was added to the cells in 1 ml DMEM with 10% fetal bovine serum and antibiotic. Drugs were used at the following final concentrations: nocodazole 20 μ M, taxol 10 μ M, latrunculin B 2.5 μ M, and jasplankinolid 125 nM. Two hours after electroporation, cells were lysed in Promega lysis buffer and luciferase activity was measured using the Promega Luciferase Assay System, as previously described [20]. All luciferase measurements were normalized to total cell protein and reported as fold increases in expression over DMSO (vehicle-treated). All experiments were performed in triplicate wells and the experiments were repeated at least three times. For statistical analyses, Student's *t* tests were used.

Plasmids. Plasmids pCMV-Lux-DTS and pCMV-GFP-DTS express either luciferase or GFP from the CMV immediate early promoter and contain the SV40 DNA nuclear targeting sequence, downstream of the reporter gene [21]. The NF κ B-responsive luciferase plasmid, pNF κ B-Luc, was from Clontech (BD Biosciences, Mountain View, CA, USA). A derivative of pCMV-GFP-DTS that contains tandem binding sites for the GeneGrip Cy3-labeled PNA (Gene Therapy Systems, San Diego, CA, USA) was used for live cell imaging and was labeled with Cy3-PNA as described [22]. All plasmids were purified from *Escherichia coli* using Qiagen Gigaprep kits as described by the manufacturer (Qiagen, Chatsworth, CA, USA).

Real-time quantitative PCR. Quantitative, real-time PCR was performed in a 20- μ l reaction volume, using the DyNAmo SYBR Green qPCR Kit as described by the manufacturer (Finnzymes, Espoo, Finland). Reactions

were carried out and quantified with the MJ Research Opticon 2. The supernatants and pellets from the microtubule and actin spin-down assays were diluted 1:1 in water and 4 μ l was used for the reactions. The primers amplified a 116-bp region of the β -lactamase gene present in the plasmids. Standard curves were generated using seven 10-fold dilutions of pCMV-Lux-DTS. The threshold was set manually by determining the best fit line for the quantitation standards. All samples were run in duplicate and amounts were determined based on the standard curve. A melting curve analysis was performed to ensure reaction specificity. Data were normalized by using the percentage DNA found in the pellet versus percentage DNA in the supernatant. All experiments were performed at least three times.

Microinjection. TC7 cells were grown on coverslips and microinjected with pCMV-GFP-DTS (0.5 mg/ml in phosphate-buffered saline) using an Eppendorf Femtojet system as previously described [22]. Drugs were added immediately following microinjection (20 μ M nocodazole or 10 μ M taxol) and maintained in the medium throughout the course of the experiment. GFP-expressing cells were counted 5 h after microinjection.

Microtubule spin-down assay. Tubulin protein, 5 mg/ml (Cytoskeleton, Inc., Denver, CO, USA) was placed on ice and 2.5 μ l of PEM buffer (80 mM Pipes, pH 7.0, 1 mM MgCl₂, 1 mM EGTA, and 50% glycerol) was added. The tubulin was allowed to polymerize for 20 min at 35°C and then stabilized by the addition of 20 μ M taxol (Cytoskeleton, Inc.). Tubulin (10 μ g) was incubated with DNA (10 ng) and/or cell extract (12–24 μ g) in PEM buffer containing 20 μ M taxol for 30 min, placed over a cushion of PEM with 50% (vol/vol) glycerol and taxol (20 μ M) added, and centrifuged at 100,000g for 40 min in an Airfuge (Beckman Instruments, Inc., Palo Alto, CA, USA). The pellet was resuspended in cushion buffer for PCR. Cell extract was prepared as described [23].

Actin spin down. Nonmuscle actin (250 μ g; Cytoskeleton, Inc.) was resuspended to a final concentration of 1 mg/ml in General Actin Buffer (Cytoskeleton, Inc.) and incubated on ice for 30 min. After incubation, 25 μ l of Actin Polymerization Buffer (Cytoskeleton, Inc.) was added and the actin was incubated for 1 h at room temperature. Reactions containing actin, plasmid DNA (10 ng), and/or cell extract were centrifuged for 1.5 h at 150,000g, the supernatant was removed, and the pellet then resuspended in an equal volume of buffer and quantitative PCR was performed.

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