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Import of Plasmid DNA into the Nucleus Is Sequence Specific

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Nuclear import of plasmid DNA in nondividing cells is a process essential to the success of numerous viral life cycles, gene therapy protocols, and gene expression experiments. Here, intact protein-free SV40 DNA was cytoplasmically injected into cells and its subcellular localization was followed by *in situ* hybridization. SV40 DNA localized to the nucleus consistent with a mechanism of transport through the nuclear pore complex (NPC): import was inhibited by the addition of the NPC-inhibitory agents wheat germ agglutinin and an anti-nucleoporin antibody as well as by energy depletion. DNA transport appeared to be a multistep process with the DNA accumulating at the nuclear periphery before its import. Most importantly, nuclear import was sequence specific: a region of SV40 DNA containing the origin of replication and the early and late promoters supported import, whereas bacterial sequences alone and other SV40-derived sequences did not. The majority of the imported DNA colocalized with the SC-35 splicing complex antigen, suggesting that the intranuclear DNA localizes to areas of transcription or message processing. This link to transcription was strengthened by the finding that inhibition of transcription blocked DNA import but not protein nuclear import. Taken together, these results support a model in which plasmid DNA nuclear import occurs by a mechanism similar to that used by nuclear localization signal-containing proteins but is also dependent on transcription.

INTRODUCTION

Intracellular macromolecular transport into and out of the nucleus occurs through the nuclear pore complex (NPC; for recent reviews see Refs [1, 2]). Microinjection and digitonin-permeabilized cell experiments have led to a model for protein nuclear import in which a nuclear localization signal (NLS)-containing protein is bound by a cytoplasmic heterodimeric protein complex, termed either karyopherin α/β or importin α/β [3–7]. This complex then docks at the NPC where the GTPase Ran and NTF2 facilitate the translocation of the NLS–protein complex into the nucleus [8–10]. This model fully supports previous findings that protein nuclear import is a two-step process: the energy-independent binding of substrate to the NPC, and the energy-requiring translocation reaction [11, 12]. Certain RNAs including the viral genomic RNAs from influenza A and several of the U snRNAs appear to use the same pathway for their import into the nucleus [13–17]. However, other pathways that may share common proteins and factors do exist [15, 18–21].

In contrast to what is known about protein and snRNP nuclear entry, the mechanism of entry of exogenous DNA into the nucleus is largely unknown. All studies involving recombinant eukaryotic gene expression rely on the ability of the introduced gene to become nuclear for function, regardless of how the DNA is introduced into the cell. Nuclear localization of extrachromosomal DNA is also important in a number of viral life cycles [22, 23]. In the case of cells undergoing cell division, migration of exogenous DNA into the nucleus is conceptually simple: since the nuclear envelope is absent during mitosis, there is no barrier between the cytoplasm and nucleus. However, in cells not actively dividing, the mechanisms of DNA nuclear import are less clear. While the nuclear import of exogenous DNA has been addressed in several experimental systems, including that of the single-stranded Ti-DNA from *Agrobacterium tumefaciens* in tobacco [24, 25] and of the HIV preintegration complex (PIC) in quiescent T cells [26–28], no general mechanisms have been elucidated.

It has recently been shown that plasmid DNA injected into multinucleated rat myotubes becomes nuclear within 10 to 20 h as assayed by β-galactosidase activity expressed from a *lacZ* gene encoded by the injected plasmids [29]. This expression was inhibited by wheat germ agglutinin (WGA), suggesting import through the NPC [30]. However, gene expression was unaffected by energy depletion, complicating the role of the NPC in this nuclear "import." Further, when the DNA was labeled with biotin and injected into the cells, no nuclear DNA was detected. Similarly, only small numbers of colloidal gold particles, representing gold-labeled DNA, could be detected in the nuclei of a few of the injected cells. An intrinsic problem to many of these experiments is that gene expression is a measure

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not only of DNA nuclear localization, but also of transcription factor nuclear import, transcription, mRNA processing and export, and translation. Thus, the effects of agents that alter any of these processes could be misinterpreted as directly modulating DNA import.

In this report, the nuclear import of plasmid DNA was followed directly using fluorescence *in situ* hybridization. An advantage of this detection approach is that it does not rely on the transcriptional activity or post-transcriptional processes that must occur in order to detect the expression of a gene product as an indicator of DNA nuclear import. Since most vectors used in eukaryotic expression studies contain sequence elements from the DNA tumor virus SV40, this 5.2-kb closed-circular DNA was chosen as a prototype eukaryotic plasmid [31]. When the DNA was microinjected into the cytoplasm of a variety of epithelial cells, it migrated to the nucleus in a time- and sequence-dependent manner and by a pathway consistent with entry through the NPC. Our results further indicate that transcription is needed for the nuclear import of plasmid DNA.

MATERIALS AND METHODS

Plasmids and viruses. The plasmid pBR-ori, containing the SV40 origin region from SV40 nt 5240 to 294, was constructed by subcloning a 351-bp *Sal*I–*Avr*II fragment from pSG5 (Stratagene, La Jolla, CA) in between the *Sal*I and *Nhe*I sites of pBR322. The plasmid pBR-polyA which contains the SV40 large T antigen polyadenylation signal (nt 2537 to 2668) was constructed by subcloning a 160-bp *Sal*I–*Eco*RI fragment from pSG5 into the *Sal*I and *Eco*RI sites of pBR322. The resulting plasmids pBR-ori and pBR-polyA are 4291 and 3870 bp, respectively. Plasmid DNA was purified by either alkaline lysis and subsequent CsCl gradient centrifugation or Qiagen midiprep columns (San Diego, CA). DNA purified in either manner displayed the same intracellular distribution after cytoplasmic microinjection. SV40 DNA was purified from infected TC7 cells as described [32].

Cell culture and microinjection. TC7 and Vero cells, sublines of African Green monkey kidney epithelium, HeLa, and HEp-2 cells were grown on coverslips in DMEM containing 10% fetal bovine serum and were cytoplasmically microinjected as described [33]. CHO cells were grown in McCoy's medium containing 10% fetal bovine serum and similarly microinjected. Purified protein-free DNA was suspended in phosphate-buffered saline and injected at a concentration of 0.5 mg/ml. Assuming that 0.1 pl is delivered by microinjection [34], this corresponds to approximately 8000 molecules of plasmid injected per cell. Rhodamine-labeled BSA–NLS [19] was injected similarly at a concentration of 0.5 mg/ml in PBS.

For energy depletion studies, the cells were incubated for 8 h after microinjection in Hanks' balanced salts solution containing 6 m*M* 2-deoxyglucose and 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). For transcription and translation inhibition studies, the cells were preincubated for 30 min in DMEM containing 10% fetal bovine serum and actinomycin D (10 μg/ml), α-amanitin (5 μg/ml), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; 20 μg/ml), or cycloheximide (50 μg/ml). The cells were microinjected and subsequently incubated in the presence of the drug for 8 h at 37°C. The concentrations of co-injected agents were 0.5 mg/ml WGA and concanavalin A (ConA) and 5 mg/ml mAb414 and IgGκ2a (Sigma, St. Louis, MO). Purified mAb414 antibody was a generous gift from D. Goldfarb (University of Rochester). In all cases, the cells shown are representative of between 50 and 400 visualized cells in at least three independent experiments.

In situ hybridization and indirect immunofluorescence. In situ hybridizations were performed as described [35], but with the following changes. After microinjection and incubation for the appropriate time, the cells were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline at 23°C for 1 min, fixed in acetone:methanol (1:1) at −20°C for 5 min, and incubated in 70% formamide in 2× SSC at 70°C for 2 min to denature the DNA. The cells were then hybridized overnight at 37°C with a fluorescently labeled probe. All samples were treated with RNaseH (8 U/ml) after hybridization and the subsequent washing steps, and the cells were mounted with DAPI and the anti-bleaching reagent DABCO. Fluorescently labeled probes were prepared by nick translation of pBR322 and SV40 DNA as described [35] except that fluorescein-12-dUTP or Texas red 5-dUTP (Molecular Probes, Eugene, OR) was incorporated directly into the DNA.

After *in situ* hybridization, anti-SC-35 hybridoma supernatant (provided by T. Maniatis, Harvard University) or a purified anti-large T antigen monoclonal antibody (Chemicon, Temecula, CA) was reacted with the cells for 2 h at 37°C followed by a TRITC-conjugated secondary antibody. All photographs were taken with an Olympus BMAX50 epifluorescence microscope equipped with a PM20 photodocumentation system on 400 ASA Kodak Ektachrome or TriX-PAN film. Confocal microscopy was performed on an ACAS 570 laser-scanning confocal microscope.

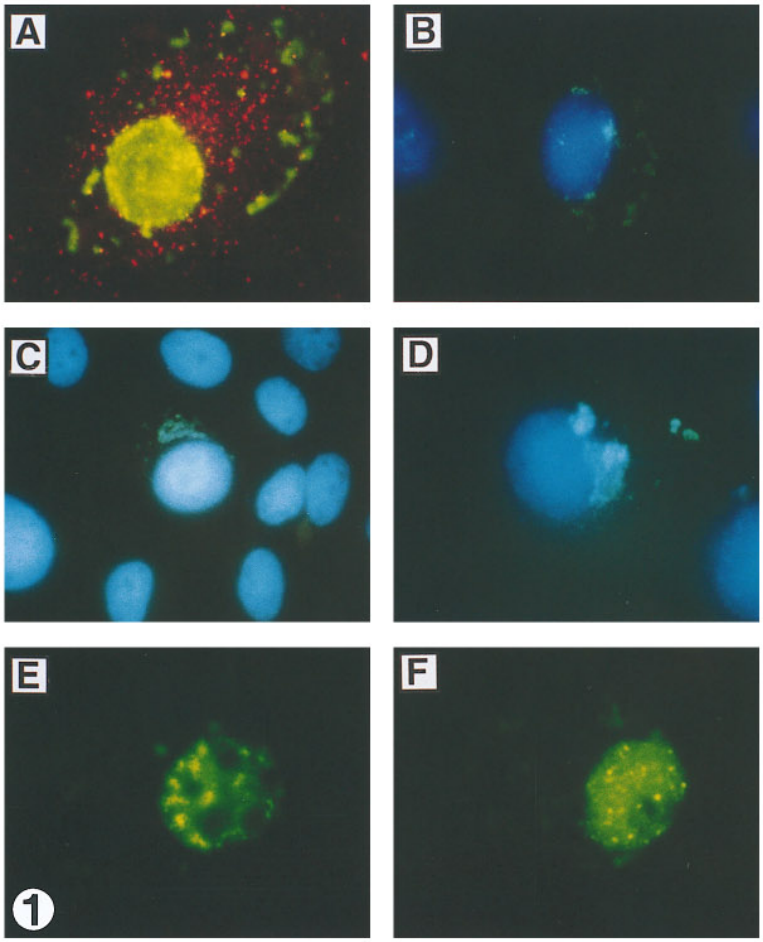
RESULTS

Nuclear Entry of Plasmid DNA Occurs in the Absence of Cell Division

To characterize the nuclear import of exogenous plasmid DNA by direct means, protein-free SV40 DNA was microinjected into the cytoplasm of TC7 African Green

FIG. 1. NPC-mediated nuclear import of DNA. (A) SV40 DNA accumulates in the nucleus of cytoplasmically injected cells. TC7 cells were cytoplasmically co-injected with rhodamine–BSA (red), a nonnuclear protein, and approximately 8000 protein-free DNA molecules and allowed to grow in complete medium for 8 h. After fixation, *in situ* hybridizations were performed with fluorescein–dUTP-labeled nick-translated SV40 DNA as probe (green). (B) ATP-depletion inhibits DNA nuclear localization. Cells were injected with DNA and incubated in the presence of 2-deoxyglucose and FCCP for 8 h, and the DNA was visualized as in A. (C) WGA and (D) mAb414 prevent nuclear accumulation of DNA. Cells were co-injected with DNA and either WGA or mAb414 and *in situ* hybridizations were performed to localize the DNA. In contrast, the non-NPC-inhibitory agents (E) ConA and (F) IgGκ2a had no effect on DNA nuclear import. The nuclei in B, C, and D are counterstained with DAPI (blue).

FIG. 5. DNA nuclear import is sequence specific. (A) pBR322 and SV40 DNA were co-injected into the cytoplasm of cells and 8 h later their locations were monitored. All of the pBR322 signal (red) remained cytoplasmic while that of SV40 (green) became nuclear. (B) pBR-ori (green), containing the SV40 early promoter and origin was imported, while (C) pBR-polyA (green) and (D) pSV small (green) were not. The nuclei in C and D are counterstained with DAPI (blue).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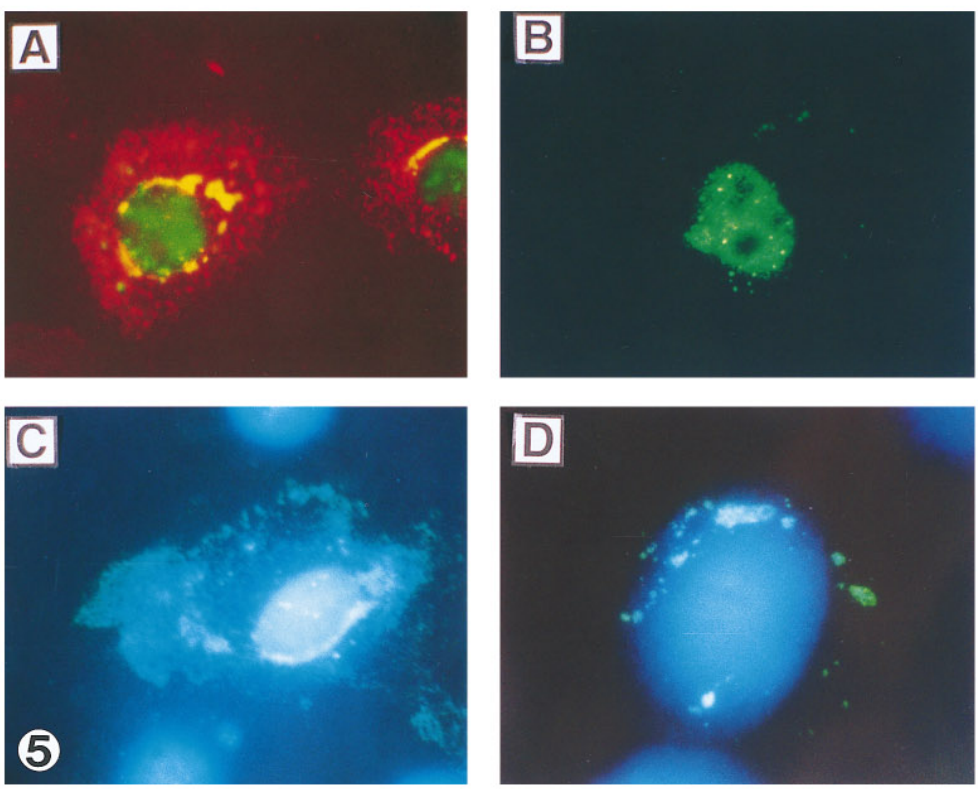
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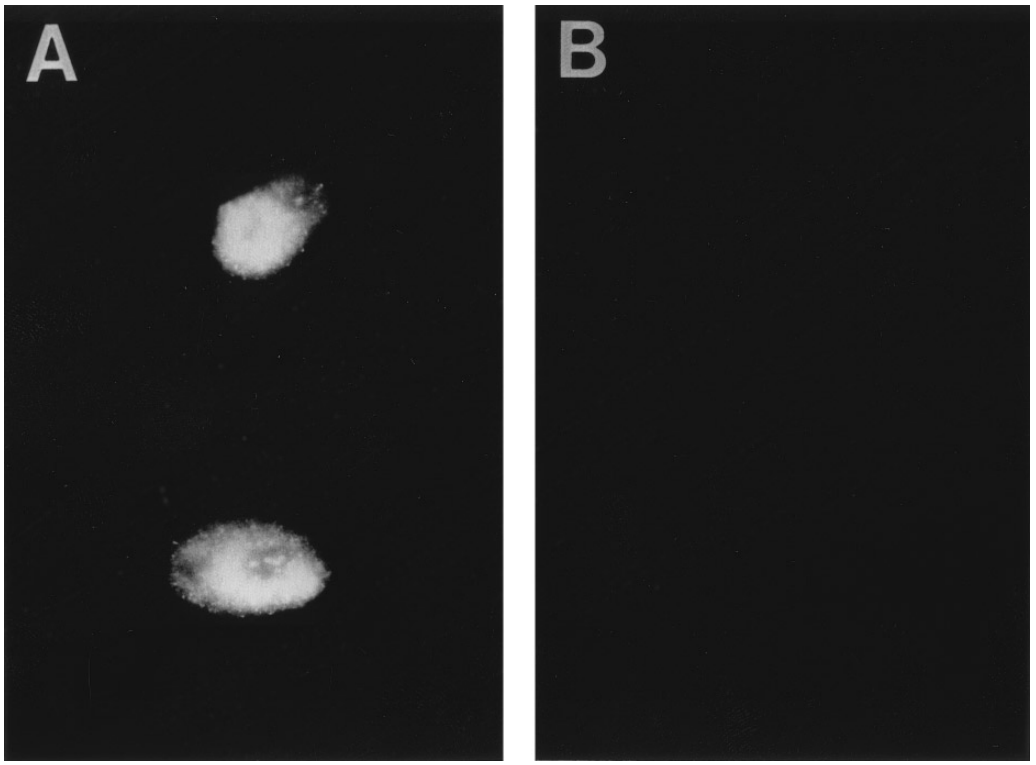

FIG. 2. RNaseH treatment effectively destroys fluorescent DNA–RNA hybrids. Nuclei of TC7 cells were microinjected with *in vitro*-transcribed SV40 RNA (4 mg/ml in DEPC-treated H_2O) and immediately fixed with either 4% paraformaldehyde in PBS for 30 min (A) [35] or methanol:acetone for 5 min at −20°C (B). After *in situ* hybridization using fluorescein–dUTP-labeled SV40 DNA and washes, the cells were either mounted (A) or treated with RNaseH (8 U/ml) as described under Materials and Methods (B).

monkey kidney epithelial cells and localized by *in situ* hybridization [35]. By 8 h after injection, the majority of the SV40 DNA was localized in the nucleus (Fig. 1A). To ensure that the signal represented the injected DNA as opposed to template-derived transcripts, the hybridized samples were treated with RNaseH to degrade any DNA–RNA hybrids [35]. The success of the RNaseH treatment was confirmed by its ability to destroy the hybridized signal of nuclear-injected *in vitro*-transcribed SV40 RNA (Fig. 2). Treatment of DNA-injected cells with RNase A to degrade total cellular RNA resulted in the same hybridization staining pattern as was seen with RNaseH treatment, confirming that the detected signal in our experiments is indeed that of the injected DNA (data not shown). Microscopic observation of the injected cells indicated that the majority had not undergone cell division and the accompanying breakdown of the nuclear envelope. This conclusion was confirmed by the fact that rhodamine-labeled BSA co-injected with the DNA remained in the cytoplasm of the injected cells while the DNA became nuclear (Fig. 1A).

Confocal microscopy was performed on the injected cells and verified that the DNA was indeed accumulating inside the nucleus as opposed to binding to the outside surface of the nuclear envelope. Eight hours after injection of SV40 DNA into the cytoplasm of TC7 cells, the DNA was localized to the nucleoplasm as seen in 0.5-μm slices through the cells (Fig. 3A). That the DNA was inside the nucleus was also confirmed by its ability to direct gene expression. Immunofluorescence was performed on cytoplasmically injected cells to detect whether they were capable of expressing large T antigen (Fig. 3B). Cells showing nuclear localization of the injected DNA by *in situ* hybridization also showed nuclear T antigen expression.

Time course studies revealed that the DNA was distributed throughout the cytoplasm at early times after injection (less than 4 h; Figs. 4A and 4D), but by 4 to 6 h, a distinct perinuclear localization of the DNA signal was seen (Fig. 4B). By 6 to 8 h, between 50 and 100% of the cells showed nuclear staining of the DNA, and in the cells that displayed nuclear DNA staining, between 60 and 100% of the fluorescent signal was in the nucleoplasm (Fig. 4C). These results suggest that DNA nuclear import may be a multistep process similar to that of NLS-containing proteins, separated into nuclear envelope binding and translocation reactions.

This DNA nuclear localization appeared to be a general phenomenon of mammalian cells, rather than due

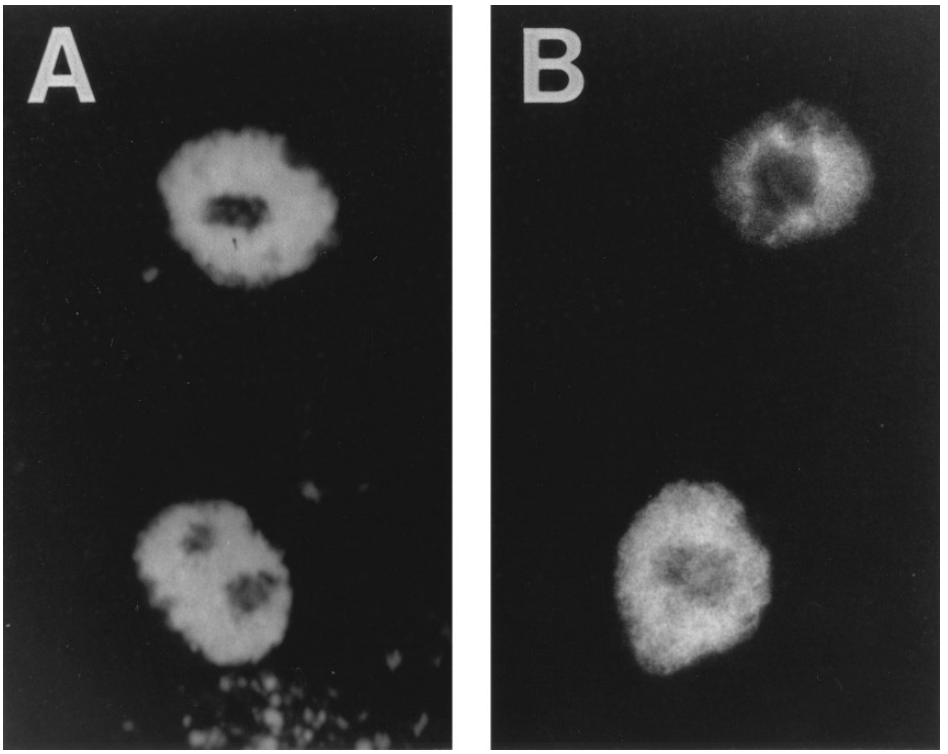

FIG. 3. Imported plasmid DNA resides in the nucleus, not bound to the nuclear envelope. TC7 cells were cytoplasmically injected with protein-free SV40 DNA, incubated for 8 h, and fixed, and *in situ* hybridization was performed as in Fig. 1. (A) Confocal microscopic localization of microinjected DNA. Successive 0.5-μm sections were visualized on an ACAS 570 laser-scanning confocal microscope. One section taken through the center of the nuclei is shown. (B) Large T antigen expression from injected DNA. After hybridization and washes, the cells were reacted with a monoclonal antibody against large T antigen.

to the permissive nature of the TC7 cells for SV40, since CHO, HeLa, HEp-2, and Vero cells (another SV40-permissive cell line) all displayed similar capacities for DNA nuclear import (Table 1).

Inhibitors of Signal-Mediated Protein Nuclear Import also Inhibit Nuclear Localization of Plasmid DNA

Transport of NLS-containing proteins into the nucleus can be inhibited by agents that are thought to occlude the NPC. These include the lectin WGA and antibodies against nucleoporins [30, 36, 37]. When either WGA or the anti-nucleoporin antibody mAb414 was co-injected with SV40 DNA into the cytoplasm of TC7 cells, the DNA remained cytoplasmic at 8 h postinjection (Figs. 1C and 1D, respectively). Co-injection of a control lectin, ConA (Fig. 1E), which does not inhibit NLS-mediated nuclear protein import, or a control isotypic mouse antibody, IgGκ2a (Fig. 1F), had no effect on the ability of SV40 DNA to localize to the nucleus.

DNA was also excluded from the nucleus when the nucleotide triphosphate pool was depleted in the cells. After injection of DNA, the cells were incubated in Hanks' balanced salts solution containing 2-deoxyglucose and FCCP to inhibit both glycolysis and oxidative phosphorylation. At 8 h, all of the injected DNA remained in the cytoplasm (Fig. 1B), whereas in energy-replete cells the DNA was nuclear (Fig. 1A). These results are all consistent with a model in which the injected DNA enters the nucleus through the NPC.

DNA Nuclear Import Is Sequence Specific

In addition to SV40 DNA, the plasmid pBR322 was also injected into the cytoplasm of cells and its subcellular localization was followed over time. Unexpectedly, in cells co-injected with both pBR322 and SV40 DNA, pBR322 (Fig. 5A, red signal) remained completely cytoplasmic while the SV40 DNA (Fig. 5A, green signal) migrated to the nucleus as in cells injected with SV40 DNA alone (Table 2). The plasmid pBR322 also remained cytoplasmic in cells injected with this plasmid alone (Table 2). This cytoplasmic compartmentalization of pBR322 was constant even at 12 h postinjection. It has been demonstrated that the rate of nuclear import of nonnuclear proteins and dextrans is inversely proportional to their size [38]. However, the difference in compartmentalization of the two DNAs was not due to a difference in size since pBR322 is actually 25% smaller than the SV40 genome. To ensure that a

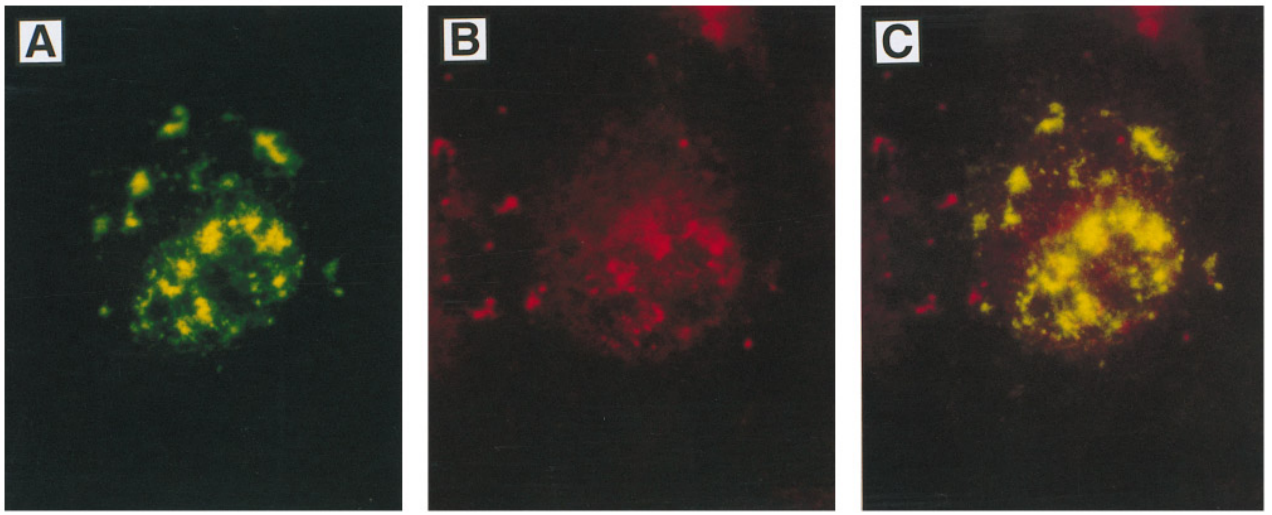

FIG. 6. SV40 DNA and the splicing center antigen SC-35 colocalize. TC7 cells were microinjected with SV40 DNA as in Fig. 1. After *in situ* hybridizations were performed at 8 h postmicroinjection, the cells were reacted with an antibody against SC-35. (A) SV40 DNA (green), (B) anti-SC-35 (red), and (C) dual exposure of A and B.

pBR322-specific signal could be detected if the DNA was nuclear, pBR322 was injected directly into the nuclei of TC7 cells and, as expected, the staining pattern was nuclear (data not shown). In addition to pBR322, pBluescript (SK^+) (Stratagene), which contains bacterial and bacteriophage sequences, also remained in the cytoplasm of microinjected cells (Table 2).

To identify the sequence(s) within the SV40 genome responsible for its nuclear localization, potential regions of the genome were subcloned into pBR322 and tested for their ability to support import into the nucleus. We reasoned that the likely sequence accounting for the difference between the SV40 and the bacterial DNA nuclear localization were the eukaryotic promoters, origins, and other *cis*-acting sequences that are not found in the bacterial plasmids. pBR-ori, containing the SV40 origin of replication and the majority of the early and late promoters, localized to the nucleus to approximately the same extent as did the intact SV40 DNA (Fig. 5B and Table 2). In contrast, pBR-polyA, containing the T antigen polyadenylation signal, and pSVsmall, a pBR322-SV40 hybrid plasmid lacking the early and late promoters as well as the origin of SV40 [33], remained in the cytoplasm (Figs. 5C and 5D, respectively). Thus, these results indicate that plasmid DNA nuclear import is mediated by specific *cis*-acting sequences.

Localization of Plasmid DNA to the Nucleus Requires Transcription

Once the cytoplasmically injected DNA entered the nucleus, there were two patterns of staining of the hybridized SV40 DNA: a diffuse staining throughout the nucleoplasm (but excluding the nucleoli) and a speckled pattern consisting of 10 to 30 foci within the nucleus (see Figs. 1A, 1E, 4C, and 6A). Similar staining patterns are obtained when cells are reacted with antibodies against splicing complex proteins (i.e., SC-35 [39, 40]). When both the microinjected SV40 DNA (Fig. 6A) and the splicing antigen SC-35 (Fig. 6B) were visualized by *in situ* hybridization and immunofluorescence, respectively, many of the foci colocalized (Fig. 6C), suggesting that much of the intranuclear plasmid DNA is localized to areas of active transcription and message processing.

Since sequences involved in transcription were necessary for DNA nuclear import, we also tested whether active transcription was a requirement for DNA nuclear import. When cells cytoplasmically injected with SV40 DNA were treated independently with agents that act by different mechanisms to inhibit transcription, the DNA remained in the cytoplasm (Fig. 7 and Table 3). Treatment of the injected cells with actinomycin D (Fig. 7C), α-amanitin (Fig. 7E), or DRB (Fig. 7G) all showed the same effect. In contrast, inhibition of protein synthesis by treatment of the cells with cycloheximide did not significantly diminish the ability of SV40 DNA to localize to the nucleus (Fig. 7I).

Based on these results, transcription appeared required for DNA nuclear import while translation did not. Alternatively, it is possible that actinomycin D, DRB, and α-amanitin acted to deplete the intracellular pool of NLS receptors or other proteins involved in nuclear import, more than cycloheximide. In this case, a defect in protein nuclear import should also be detected. To test this, a rhodamine-labeled NLS peptide–

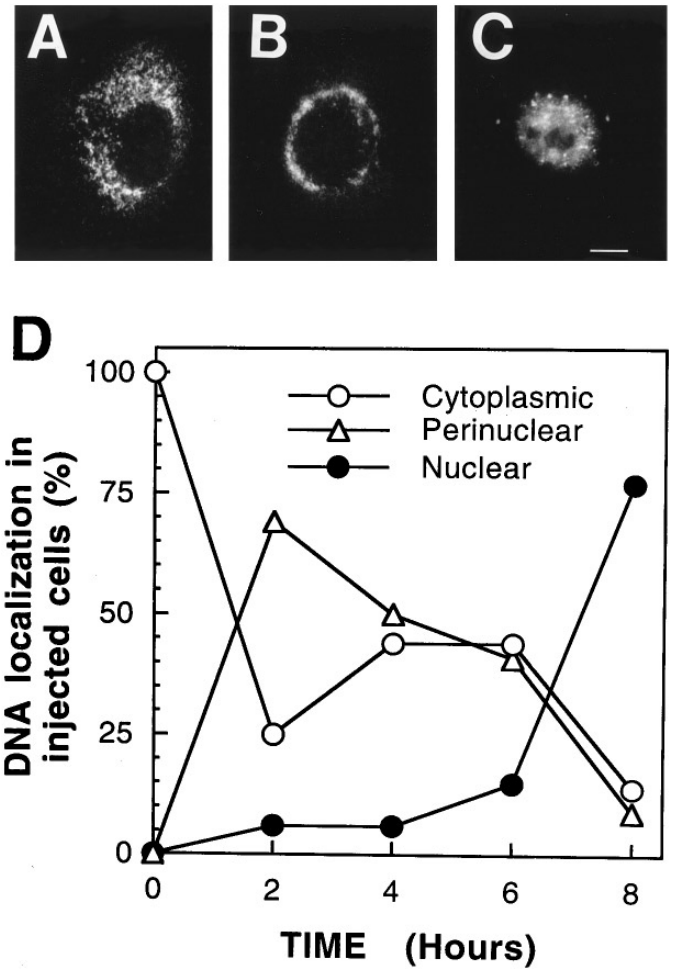

FIG. 4. Time course of DNA nuclear import. TC7 cells were cytoplasmically injected with protein-free SV40 DNA, incubated for the indicated times, and fixed, and the location of the injected DNA was determined. Cells with cytoplasmic (A), perinuclear (B), or nuclear (C) DNA staining were counted and the percentages of cells with each staining pattern are shown (D). Between 50 and 100 cells were counted for each time point. Bar, 10 μm.

BSA conjugate was injected into the cytoplasm of cells after they had been treated with inhibitors for 7 h, and its nuclear localization was assessed 1 h later (Figs. 7B, 7D, 7F, 7H, and 7J, and Table 3). Thus, if these agents are depleting intracellular protein pools needed for general nuclear import, by 7 h the effect should be evident. While DNA nuclear import was inhibited effectively by inhibitors of transcription but not by cycloheximide, none of the inhibitors decreased protein nuclear import.

DISCUSSION

While considerable progress has been made in recent years in characterizing the signals and proteins involved in protein and RNA nucleocytoplasmic trafficking, very little is known about the nuclear import of exogenous plasmid DNA. In this report, we have begun to characterize this transport process. Using *in situ* hybridization to localize the substrate DNA directly, we have demonstrated that protein-free plasmid DNA can enter the nuclei of nondividing mammalian cells. The inhibition of import by the anti-nucleoporin antibody mAb414, the lectin WGA, and energy depletion supports a model in which the nuclear entry of plasmid DNA occurs through the NPC. Similar to protein and snRNP nuclear import, the import of plasmid DNA is signal-mediated, requiring the presence of an RNA polymerase II promoter and/or an origin of replication. Finally, a role for transcription in the nuclear import of DNA was implicated by two findings. First, the subnuclear localization of the imported DNA coincided with that of a splicing complex antigen, SC-35, indicating that the imported DNA migrates to intranuclear areas of transcription and mRNA

TABLE 1

DNA Nuclear Import Occurs in Multiple Cell Types

Cell line	Nuclear localization (% injected cells)[a]			
	2 hpi[b]	4 hpi	6 hpi	8 hpi
TC7	6	6	15	78
Vero	0	0	32	54
CHO	ND[c]	ND	ND	63
HeLa	19	11	60	68
Hep-2	16	30	50	70

[a] Cells were cytoplasmically microinjected with purified SV40 DNA (0.5 mg/ml) in PBS and incubated for the indicated times. *In situ* hybridizations were performed as described under Materials and Methods. At least 200 cells were injected in each experiment.
[b] Hours postinjection.
[c] Not done.

TABLE 2

Nuclear Localization of SV40 Hybrid Plasmids

Plasmid DNA	Nuclear localization (% injected cells ± SD)[a]
SV40	81 ± 12
pBR322	0 ± 1
bBluescript (SK$^+$)	0 ± 0
pSVsmall	0 ± 0
pBR-polyA	2 ± 1
pBR-ori	62 ± 17

[a] TC7 cells were cytoplasmically microinjected with purified plasmid DNA (0.5 mg/ml) in PBS and incubated for 8 h. *In situ* hybridizations were performed using a 1:1 mixture of fluorescein-labeled SV40 and pBR322 DNA and as described under Materials and Methods. At least 100 cells were injected for each plasmid and the results are the means of three experiments ± SD.

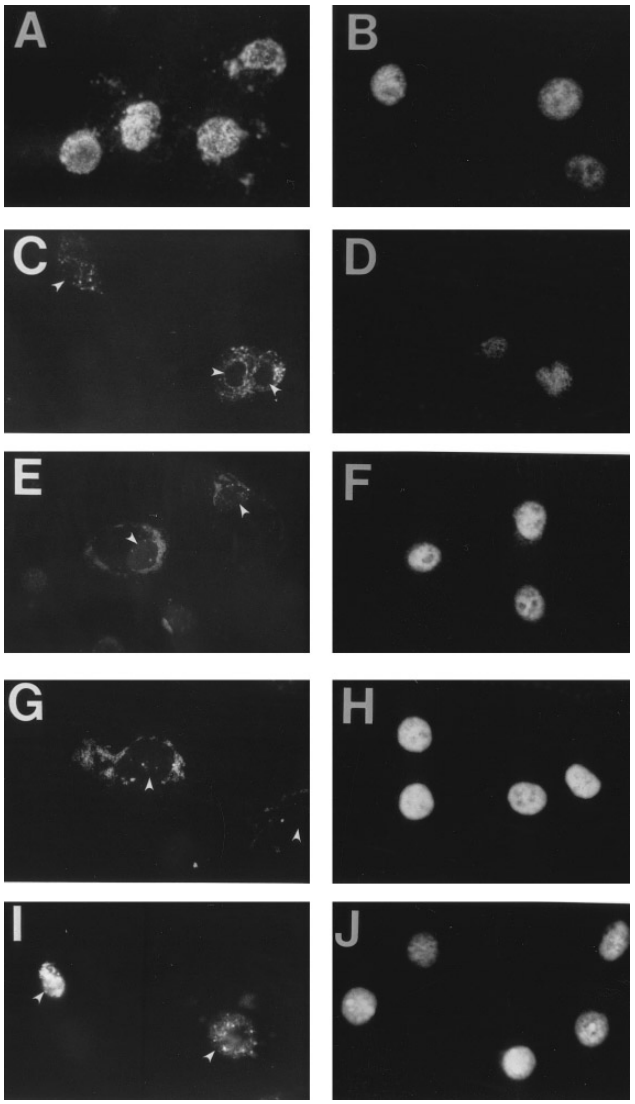

FIG. 7. Effects of transcription and translation inhibition on DNA and protein nuclear import. TC7 cells were pretreated for 30 min, cytoplasmically injected with SV40 DNA, and incubated for 8 h in the absence (A and B) or presence of actinomycin D (10 μg/ml; C and D), α-amanitin (5 μg/ml; E and F), DRB (20 μg/ml; G and H), or cycloheximide (50 μg/ml; I and J). Seven hours post-DNA-injection, rhodamine-labeled BSA–NLS (0.5 mg/ml) was cytoplasmically injected into other cells on the same coverslips. At 8 h, the cells were fixed, *in situ* hybridizations were performed to visualize the SV40 DNA (A, C, E, G, and I), and the rhodamine-labeled BSA–NLS was detected directly (B, D, F, H, and J). Arrowheads indicate the location of nuclei of injected cells.

processing, and second, the inhibition of RNA polymerase II-mediated transcription caused the DNA to remain in the cytoplasm.

Nuclear import of NLS-containing proteins is a relatively rapid process. Under optimal conditions in either microinjected or digitonin-permeabilized cells, 5- to 20-fold nuclear accumulation of protein is seen within 30 min [41, 42]. In contrast, nuclear import of DNA was not detected at significant levels until 6 to 8 h postmicroinjection. The most likely reason for the much slower rates of DNA nuclear import is due to the large size of the imported DNA. Protein nuclear transport has been demonstrated to be inversely proportional to the size and shape of the imported protein [43, 44]. While a 465-kDa NLS-protein was imported completely within 2 h of injection, only a fraction of a 970-kDa NLS-protein has been transported by the same time [43]. Since the 5.2-kb SV40 DNA is clearly larger than either of these proteins, the length of time required for import is expected to be longer.

The inhibition of plasmid DNA nuclear localization by WGA, mAb414, and energy depletion, all of which also inhibit NLS-dependent, NPC-mediated protein nuclear import, suggests that exogenous DNA uses a pathway similar to that used by NLS-containing proteins and certain RNPs [11, 12, 15, 17, 30, 36]. Thus, it is likely that the DNA is being complexed by proteins in the cytoplasm that contain NLSs and which will act as a bridge between the DNA and NLS-receptors. Based on our finding that the origin/promoter region of SV40 is required *in cis* for DNA nuclear import, the most likely candidates for these bridging factors are transcription factors and origin-binding proteins. Indeed, this region of SV40 DNA contains numerous consensus binding sites for a multitude of transcription factors, including SP1, AP1, Oct-1, and NF-κB [45]. Since these proteins contain NLSs to facilitate their nuclear targeting and bind DNA, they may interact with both the NLS receptor complex and cytoplasmic plasmid DNA on their way to the nucleus. However, while the NLS-mediated pathway may be used for the nuclear import of DNA, it is not sufficient based on the DNA-specific inhibitory effects of transcription inhibitors.

A similar bridging mechanism has been proposed to occur in the nuclear import of influenza A RNPs. Using permeabilized cells, it was shown that the viral RNAs

TABLE 3

Nuclear Import of Plasmid DNA and NLS–BSA Shown in Fig. 7

	Nuclear localization (% injected cells ± SD)[a]	
Plasmid DNA	SV40 DNA	Rhodamine–BSA–NLS
Control	87 ± 12	100 ± 0
Actinomycin D	0 ± 0	94 ± 8
α-Amanitin	0 ± 0	88 ± 17
DRB	3 ± 4	100 ± 0
Cycloheximide	59 ± 18	97 ± 4

[a] At least 100 cells were injected for each experiment (DNA and BSA–NLS) and the results are the means of three experiments ± SD.

could be imported only in the presence of both the karyopherin α and β/RAN/p10 complexes, which are required for protein nuclear import, and the viral nucleocapsid proteins, which bind to the viral RNA and contain an NLS [17]. In experiments designed to address the role of capsid proteins in targeting infectious SV40 virions to the nucleus, it has been demonstrated recently that the presence of viral capsid proteins which contain NLSs [46, 47] and bind DNA [48] greatly stimulates the time course for nuclear accumulation of microinjected viral DNA [49]. When capsid proteins, supplied in the form of DNA-lacking empty viral particles, were co-injected with SV40 DNA, the time course for viral T antigen expression increased by a factor of 2, suggesting that the SV40 capsid proteins are acting to target the DNA to the nucleus. A role for viral proteins has also been postulated for the nuclear import of HIV PICs [26–28]. Nuclear localization of the HIV PIC depends on the presence of a functional NLS within the matrix protein present in the PIC [27, 28]. The inability of a mutant NLS-containing matrix protein to localize the HIV DNA-containing complex to the nucleus suggests either that the HIV PIC does not utilize the cellular pathway described here, perhaps due to the sequence differences between the SV40 origin region and the HIV LTR promoter, or more likely that the quiescent T cells in which the HIV experiments were performed do not express the cellular factors necessary for plasmid DNA nuclear import, either at the appropriate time or at the necessary levels, and hence the virus must provide its own.

We have identified a region of SV40 DNA that is sufficient for promoting the nuclear entry of a nonnuclear localizing plasmid in cells that are not dividing. This sequence contains an origin of replication, portions of two promoters, and the SV40 enhancer. Whether this sequence also promotes nuclear localization of plasmid DNA in cells actively dividing was not tested, but previous results suggests that it can. A smaller region of the SV40 genome encompassing the 72-bp enhancer element was shown to lead to increased transcription of a reporter gene in cells that were dividing [50]. As expected, the presence of the enhancer stimulated transcription of a plasmid-encoded reporter gene when compared to a similar construct lacking the enhancer. However, when the plasmids were injected into the cytoplasm and assayed for gene activity over several cell doublings, the stimulation by the enhancer was greater than could be accounted for by classic enhancer activity, suggesting that this sequence can increase nuclear localization of the reporter plasmid even in actively dividing cells.

The link between transcription and plasmid DNA nuclear localization was quite intriguing. A similar link between transcription and nuclear transport has also been observed for the import of the A1 hnRNA binding protein [51, 52]. The A1 protein has the ability to shuttle into and out of the nucleus. However, in the presence of RNA polymerase II inhibitors, A1 is not reimported into the nuclei of heterokaryons, while other nuclear proteins are transported. Thus, transcription is required for nuclear import of A1, although how is unknown. One possible explanation for the requirement of RNA synthesis could be that a specific RNA is needed for the nuclear import of these substrates. At present there is no evidence of a structural RNA playing a role in the nuclear import of proteins or ribonucleoprotein complexes, but the possibility that such an RNA is involved in DNA or certain RNP complex nuclear import has not been excluded.

How then might transcription be required for DNA nuclear import? Since the sequence necessary for SV40 DNA nuclear import contains two RNA polymerase II promoters, an attractive hypothesis is that RNA polymerase II is involved in the import or retention of DNA in the nucleus. While it is unlikely that RNA polymerase II is binding to the DNA in the cytoplasm and carrying it into the nucleus, it is possible that one or more of its accessory transcription factors, many of which are known to bind to the SV40 promoter region, may do so. The ability of the DNA to localize to the nucleus when protein synthesis is inhibited, however, would suggest that either these DNA binding proteins remain in the cytoplasm for up to 8 h after synthesis or that they may shuttle across the nuclear envelope. Alternatively, inhibition of protein synthesis may not be as absolute as that of transcription and may allow ongoing translation, albeit at greatly reduced levels. Although we detected no T antigen in cycloheximide-treated SV40 DNA-injected cells (not shown), we cannot rule out this possibility. That protein import was unaffected by transcription inhibition suggests that while DNA appears to utilize a pathway similar to that used by proteins for its nuclear entry, additional factors are needed for DNA nuclear import.

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