A Cytoplasmically Anchored Nuclear Protein Interferes Specifically with the Import of Nuclear Proteins but not U1 snRNA

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Abstract. A cytoplasmically anchored mutant SV40 T antigen, FS T antigen, was shown previously to interfere specifically with the nuclear import of a heterologous nuclear protein, adenovirus 5 fiber protein, in cultured monkey cells (Schneider, J., C. Schindewolf, K. van Zee, and E. Fanning. 1988. *Cell.* 54:117–125; van Zee, K., F. Appel, and E. Fanning. 1991. *Mol. Cell. Biol.* 11:5137–5146). In this report, we demonstrate that FS T antigen also interferes with the nuclear import of adenovirus E1A and a peptide-albumin conjugate bearing multiple copies of the T antigen nuclear localization signal, but not with the import of U1 snRNA. A kinetic analysis indicates that nuclear import of the albumin-peptide conjugate is inhibited only when high intracellular concentrations of FS T antigen are reached. After microinjection into the cytoplasm of cultured cells, purified FS T antigen protein does not accumulate at the nuclear periphery, but rather is distributed in a punctate pattern throughout the cytoplasm. These data support a model in which cytoplasmic anchoring of FS T antigen enables the mutant protein to sequester and titrate out a cellular factor which is required for nuclear protein but not U1 snRNA import.

EXCHANGE of molecular information between the nucleus and cytoplasm is mediated via a bidirectional trafficking pathway. Diverse macromolecules, including proteins, tRNAs, mRNAs, and snRNAs, enter and exit the nucleus through aqueous channels formed by the nuclear pore complexes (NPC)[1] (reviewed in Silver, 1991; Nigg et al., 1991). Although ions, small metabolites and small nonnuclear proteins are able to diffuse across the NPC (Bonner, 1975; Paine et al., 1975; Peters, 1986), the import of karyophilic proteins (Goldfarb et al., 1986), including nuclear proteins which are smaller than the NPC diffusion limit of 20–40 kD (Feldherr et al., 1984; Breeuwer and Goldfarb, 1990), as well as the export of tRNAs (Zasloff, 1983), mRNAs (reviewed in Izaurralde and Mattaj, 1992), and ribosomal subunits (Khanna-Gupta and Ware, 1989; Bataille et al., 1990) are strictly regulated.

The selectivity of nuclear-cytoplasmic transport is best understood for nuclear protein import. Discrete stretches of amino acids called nuclear localization signals (NLSs) have been identified which are necessary and sufficient to target proteins to the nucleus (Feldherr et al., 1984; Kalderon et al., 1984*a*; Lanford and Butel, 1984; Lanford et al., 1986; Goldfarb et al., 1986; Richardson et al., 1988; reviewed in Roberts, 1989, and Garcia-Bustos et al., 1989). Although a clear consensus sequence has not emerged (reviewed in Dingwall and Laskey, 1991), most NLSs contain either a cluster of basic amino acids flanked by a proline or glycine as seen in the NLS of SV40 T antigen (Kalderon et al., 1984*b*; Chelsky et al., 1989) or two short stretches of basic amino acids separated by a spacer, as seen in the NLS of nucleoplasmin (Dingwall et al., 1988; Robbins et al., 1991).

All of the experimental evidence to date supports the existence of a saturable, signal-specific recognition step in nuclear protein import thought to be mediated by NLS receptors (Goldfarb et al., 1986; Breeuwer and Goldfarb, 1990; reviewed in Garcia-Bustos et al., 1990; Silver, 1991). On the basis of kinetic studies in *Xenopus laevis* oocytes, the SV40 T antigen and nucleoplasmin NLSs define a single class of nuclear protein import receptors (Michaud and Goldfarb, 1991). NLS-binding proteins which may function as nuclear import receptors have been identified in a variety of species in both nuclear and cytoplasmic compartments (Adam et al., 1989; Li and Thomas, 1989; Meier and Blobel, 1990; Silver et al., 1989; Adam and Gerace, 1991; Yamasaki et al., 1989; Imamoto-Sonobe et al., 1990; Stochaj et al., 1991; Lee et al., 1991; Stochaj and Silver, 1992; reviewed in Garcia-Bustos et al., 1990, and Silver, 1991). Direct evidence that NLS-binding proteins play a role in nuclear import in vitro has been presented for two polypeptides of 54 and 56 kD isolated from bovine erythrocytes (Adam et al., 1990; Adam and Gerace, 1991). Evidence that any of these NLS-binding proteins function as nuclear import receptors in vivo is still lacking.

Recently, experiments in *Xenopus laevis* oocytes have

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1. *Abbreviations used in this paper*: bHSA, biotinylated human serum albumin; HSA, human serum albumin; NLS, nuclear localization signal; NPC, nuclear pore complex.

shown that nuclear import of U1, U2, U4, and U5 snRNPs is mediated by a pathway kinetically distinct from the pathway utilized by T antigen, suggesting the existence of a separate class of karyophilic signal receptors (Fischer et al., 1991; Michaud and Goldfarb, 1991, 1992). These snRNPs are each assembled in the cytoplasm from a small nuclear RNA and multiple protein components, including a number of common Sm proteins as well as U snRNP-specific proteins, before being transported to the nucleus (reviewed in Lührmann et al., 1990). In both somatic cells (Sauterer et al., 1988) and *Xenopus laevis* oocytes (Zeller et al., 1983), the free core proteins are synthesized in excess over U snRNA and only enter the nucleus after assembly into a mature snRNP particle (reviewed in Izaurralde and Mattaj, 1992). Two groups have demonstrated that in oocytes the nuclear targeting signals of U1 and U2 snRNPs are bipartite (Hamm et al., 1990; Fischer and Lührmann, 1990). This bipartite NLS is dependent on both the binding of the common U snRNP proteins to a single-stranded region of the U snRNA (referred to as the Sm site), as well as on the presence of a trimethyl guanosine cap structure on the snRNA (Hamm et al., 1990; Fischer and Lührmann, 1990; Fischer et al., 1991). Taken together these results suggest that multiple targeting mechanisms and classes of receptors may exist to accommodate the diversity of karyophilic transport substrates.

In contrast with the diversity of karyophilic signal receptors, there is no evidence so far to suggest the existence of functionally distinct classes of NPCs. Electron microscopy studies have demonstrated that gold particles coated with nuclear proteins or RNA traverse the same NPCs (Dworetzky and Feldherr, 1988), and antibodies directed against components of the NPC inhibit both protein import and RNA export (Featherstone et al., 1988).

One strategy to increase our understanding of the cellular factors required for the specificity of nuclear targeting is to characterize mutant proteins which interfere with the nuclear transport apparatus. Previously, we described an SV40 large T antigen mutant, FS T antigen, which fails to accumulate properly in the nucleus although it retains a wild-type NLS and interferes in an NLS-dependent manner with the nuclear import of a heterologous protein (Schneider et al., 1988; van Zee et al., 1991). Expression of this mutant protein has lethal consequences for the cell (Schneider et al., 1988; van Zee et al., 1991). The abnormal localization and lethality of FS T antigen are species-, cell line- and tissue-independent. Introduction of a defective NLS in the FS T antigen background relieves both FS T antigen's lethality and interference with nuclear protein import, demonstrating that FS T antigen interferes specifically with the nuclear transport machinery (Schneider et al., 1988; van Zee et al., 1991). As a result of a frameshift mutation in the DNA sequences encoding the carboxy terminus of T antigen, FS T antigen bears a longer, more hydrophobic carboxy terminus than wild-type T antigen (Schneider et al., 1988). The novel carboxy terminus of FS T antigen functions as a cytoplasmic anchoring signal which overrides the SV40 T antigen NLS independently of protein context (van Zee et al., 1991).

With the goal of determining how FS T antigen interferes with the nuclear transport machinery, we have examined the influence of this mutant on distinct nucleocytoplasmic trafficking pathways. In this report, we present evidence that FS T antigen interferes specifically with the active import of heterologous nuclear proteins but not with the import of U1 snRNA. We also demonstrate that purified FS T antigen protein fails to be transported to the nucleus after microinjection into the cytoplasm and does not accumulate at the nuclear periphery.

Materials and Methods

Plasmids

pSVWT, pSVFS, and pSVcTFS were described (van Zee et al., 1991). pBSVcT.3 was kindly provided by R. Lanford (Southwest Foundation for Biomedical Research, San Antonio, TX) (Lanford and Butel, 1984). Plasmid pU1 containing a wild-type *Xenopus laevis* U1 snRNA gene under control of a T7 RNA polymerase promoter has been described (Hamm et al., 1987, 1990) and was generously provided by I. Mattaj, European Molecular Biology Laboratory, Heidelberg, Germany. The plasmids p941FS and p941cT3 were constructed by replacing a 2-kb *Pfl*M I/*Bam*H I fragment of p941T (Lanford, 1988) encoding the carboxy-terminal sequences of wild-type T antigen with the corresponding *Pfl*M I/*Bam*H I fragment from pBSVcT.3 (Lanford and Butel, 1984) or pSVFS (van Zee et al., 1991).

Cell Culture

Vero African green monkey kidney and HeLa cells were grown in DME supplemented with antibiotics and 5% (HeLa) or 10% (Vero) FCS (Biochrom K.G., Berlin, Germany) in a humidified incubator at 37°C under a 10% CO_2 atmosphere.

Microinjection

For microinjection experiments Vero or HeLa cells were plated at least 24 h before microinjection onto glass coverslips (2×10^4 cells/cm^2) scored with a grid to facilitate relocation of injected cells. A microinjector (model 5242) and manipulator (model 5170) (Eppendorf, Hamburg, Germany) mounted on an inverted IM35 microscope (Carl Zeiss, Oberkochen, Germany) were used to deliver samples. All samples were centrifuged at 13,000 *g* for 10 min before microinjection. Microinjection needles were pulled from glass capillaries (Clark Electromedical Instruments, Reading, UK) on an automatic pipette puller (David Kopf Instruments, Tujunga, CA), or purchased (Femtotips) from Eppendorf. Plasmid DNA (0.2–0.5 mg/ml) and NLS peptide-albumin conjugate (0.5–1 mg/ml as indicated in the text and figure legends) were diluted in microinjection buffer (0.048 M K_2HPO_4, 0.0045 M KH_2PO_4, 0.014 M NaH_2PO_4) (Graessmann et al., 1980). Digoxygenin-labeled U1 snRNAs and tRNA were microinjected undiluted at a concentration of ~5 mg/ml.

Adenovirus Infection

Vero cells were infected with adenovirus 5 at a multiplicity of infection of 10 for 1 h at 37°C. Virus stocks were diluted in a minimal vol of DME. After 1 h, the inoculum was removed, and the cells were washed twice with fresh medium and returned to 37°C for 8 h before fixation.

Immunofluorescent Staining

Microinjected cells were washed gently three times with phosphate buffered saline (13 mM Na_2HPO_4, 2 mM NaH_2PO_4, 5 mM KCl, 150 mM NaCl, pH 7.4) (PBS), fixed in 3% paraformaldehyde in PBS for 20 min, washed twice with PBS, and permeabilized in 0.1% Triton X-100 in PBS for 10 min. Cells were blocked in 10% FCS in PBS for at least 30 min to reduce nonspecific staining. Antibodies were diluted as indicated below in 10% FCS in PBS, and all incubations were carried out at room temperature. Cells were washed extensively with PBS after each antibody incubation. After the last wash step, cells were mounted in 90% glycerol containing 0.1 mg/ml para-phenylenediamine (Johnson and Nogueira Araujo, 1981) and viewed on a Zeiss IM35 microscope using a 63× objective.

T antigen species were visualized using either a polyclonal antiserum directed against SDS-denatured T antigen (1:1,000, 45 min) (gift of W. Deppert, Heinrich-Pette-Institut, Hamburg) followed by a Texas red-conjugated

species-specific second antibody (1:100, 45 min), with the monoclonal antibody Pab419 (10 μg/ml, 1 h) (Harlow et al., 1981) followed by a fluorescein isothiocyanate (FITC)-labeled species-specific second antibody (1:100, 45 min), or with the mAbs Pab220 and Pab221 (10 μg/ml) (Mole et al., 1987). E1A protein was stained with the mouse mAb m73 (10 μg/ml, 1 h) (Harlow et al., 1985), followed by an FITC-conjugated second antibody. Digoxygenin-labeled U1 snRNA was detected using a mouse antidigoxygenin antibody (10 μg/ml, 2 h) purchased from Boehringer Mannheim (Mannheim, Germany) followed by an FITC-labeled species-specific second antibody. Texas red-conjugated streptavidin (1:200, 45 min) was used to visualize NLS-peptide conjugates. Texas red-conjugated donkey anti-rabbit IgG (1:200; 1 h) was used to detect rabbit IgG. Secondary antibodies and Texas red-streptavidin were purchased from Amersham International (Braunschweig, Germany).

Synthesis of Digoxygenin-labeled U1 snRNA

Digoxygenin (DIG)-labeled U1 snRNA transcripts were synthesized in vitro using T7 RNA polymerase and digoxygenin-labeled UTP (Boehringer Mannheim). 5 μg of *Bam*H I-linearized DNA template was incubated in a 50 μl vol containing 40 mM Tris-HCl, pH 7.9; 10 mM NaCl; 6 mM $MgCl_2$; 10 mM DTT; 2 mM spermidine; 1 mM each ATP, CTP, and GTP; 0.65 mM UTP; 0.35 mM digoxygenin-labeled UTP (nucleotides were purchased as a 10× concentrated DIG-RNA labeling mixture from Boehringer Mannheim); 1 mM m^7GpppG cap analogue (New England Biolabs, Schwalbach, Germany); 10 U RNasin; and 30 U T7 RNA polymerase for 90 min at 37°C. The DNA template was removed by addition of 1 U/μl RNase-free DNase for 30 min at 37°C. Water was added to a final volume of 100 μl, proteins were extracted with phenol-chloroform. 18 μl of 6 M ammonium acetate and 236 μl of ethanol were added, and the RNA was precipitated by incubation at −80°C for 1 h followed by centrifugation at 4°C. RNA pellets were washed twice with 70% ethanol and resuspended in 5 to 10 μl of water. In vitro transcription buffer and all enzymes were purchased from Promega Biotec (Heidelberg, Germany).

Preparation of NLS Peptide-Biotinylated Albumin Conjugate

A peptide corresponding to the wild-type SV40 T antigen nuclear localization signal was chemically conjugated to biotinylated human serum albumin (HSA) (Calbiochem, Frankfurt, Germany) for use as a nuclear import substrate. HSA was biotinylated with biotin-N-hydroxysuccinimide ester (Life Technologies Gibco/BRL, Eggenstein, Germany) according to the manufacturer's instructions. Purified peptide (Bissendorf Biochemicals, Hannover, Germany) was conjugated to biotinylated HSA using the heterobifunctional crosslinker m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce Europe B. V., Oud Beijerland, The Netherlands) as previously described by Goldfarb et al. (1986) and modified by Silver et al. (1989). Unconjugated peptide was removed by gel filtration over a Sephadex G25 (Pharmacia Fine Chemicals, Freiburg, Germany) column equilibrated in 0.1 M NH_4CO_3. The degree of conjugation was estimated by electrophoresis on a 10% polyacrylamide gel (Laemmli, 1970).

Purification of SV40 T Antigens

Wild-type, cT.3 and FS T antigens were purified from Sf9 insect cells infected with the appropriate recombinant baculovirus. The recombinant baculovirus 941T encoding wild-type T antigen was kindly provided by Robert Lanford (Southwest Foundation for Biomedical Research, San Antonio, TX). Recombinant baculoviruses producing cT.3 and FS T antigens were isolated after cotransfection of Sf9 cells with the plasmids p941cT.3 or p941FS, respectively, and AcMNPV genomic baculovirus DNA as described (Summers and Smith, 1987). Sf9 cells were infected with 10 plaque-forming units per cell and incubated for 40 h before harvesting. Wild-type and cT.3 T antigens were purified by immunoaffinity chromatography essentially as described (Simanis and Lane, 1985; Lanford, 1988), except that the immunoabsorbent was the monoclonal antibody Pab 101 (Gurney et al., 1980) coupled to Tresyl-activated Sepharose 4B (Pharmacia LKB Biotechnology, Inc., Freiburg, Germany) (Höss et al., 1990). FS T antigen was purified in a similar way except that the immunoabsorbent Pab 419 (Harlow et al., 1981) coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Inc.) was used, and the protein was eluted using 0.1 M K_3PO_4, 20% glycerol, pH 12.8, followed by neutralization with 1/5 vol 0.5 M KH_2PO_4. Protein concentration was estimated spectrophotometrically using a calculated molar extinction coefficient of 1.2 based on the amino acid composition of T antigen (Gill and von Hippel, 1989).

Results

FS T Antigen Interferes with the Nuclear Import of Adenovirus E1A

We have previously observed in transiently transfected cells that FS T antigen interfered with the nuclear import of the adenovirus 5 fiber protein (Schneider et al., 1988) and DNA binding protein (van Zee and Fanning, unpublished data). However, we were concerned that the ability of adenovirus to shut down host protein synthesis concomitant with the onset of the late phase of the infectious cycle (Huang and Schneider, 1991; reviewed in Schneider and Shenk, 1987) and to influence the nuclear export of host mRNA molecules (Leppard and Shenk, 1989, and references therein) could be indirectly responsible for the impaired nuclear import of these late viral proteins by influencing the availability of cellular factors required for mediated protein import. Thus, we wished to determine whether FS T antigen interferes with the import of the viral nuclear protein E1A in the early phase of the infectious cycle before host protein synthesis is shut down.

In this experiment, we used microinjection to deliver plasmid DNA encoding FS or control T antigens. Cells were infected with adenovirus 5, 1 h after microinjection and incubated for an additional 8 h before fixation. This time period allowed us to examine the subcellular distribution of E1A during the early phase of the infectious cycle in cells expressing FS T antigen before the cells began to display the alterations in morphology observed at later times after microinjection of SVFS plasmid DNA (van Zee et al., 1991). In cells expressing FS T antigen (Fig. 1, *A* and *B*), E1A was distributed in the cytoplasm as well as in or over the nucleus (Fig. 1, *C* and *D*). This subcellular distribution contrasts with the completely nuclear distribution of E1A in cells not expressing FS T antigen (compare the cells in Fig. 1, *C* and *D*, with those in *G* and *H*). The failure of E1A to accumulate properly in the nucleus is dependent on FS T antigen having a wild-type NLS, as the double mutant cTFS T antigen (Fig. 1 *F*) did not interfere with the nuclear uptake of E1A (Fig. 1 *H*). Similarly, the cytoplasmic cT.3 T antigen mutant (Fig. 1 *E*) did not interfere with nuclear import of E1A (Fig. 1 *G*). These results demonstrate that FS T antigen interferes with heterologous nuclear protein import during the early phase of the adenovirus infectious cycle.

Interference with Nuclear Import of a Nonviral Protein

Next, we investigated whether FS T antigen interferes with the import of a nonviral nuclear protein, an NLS peptide-protein conjugate (designated NLS-bHSA) which contains multiple copies of a synthetic peptide containing the T antigen NLS chemically cross-linked to biotinylated human serum albumin (bHSA) (Goldfarb et al., 1986; Silver et al., 1989). The sequence of the synthetic T antigen NLS peptide used in this study is NH_2-Cys-Thr-Pro-Pro-Lys-Lys^{128}-Lys-Arg-Lys-Val-COOH (underscored amino acids comprise the T antigen NLS; 128 indicates T antigen residue position). This synthetic peptide is able to induce the nuclear transport of nonnuclear proteins after microinjection into the cytoplasm of mammalian cells and *Xenopus* oocytes (Goldfarb et al., 1986; Lanford et al., 1986, 1990; Dworetzky et al.,

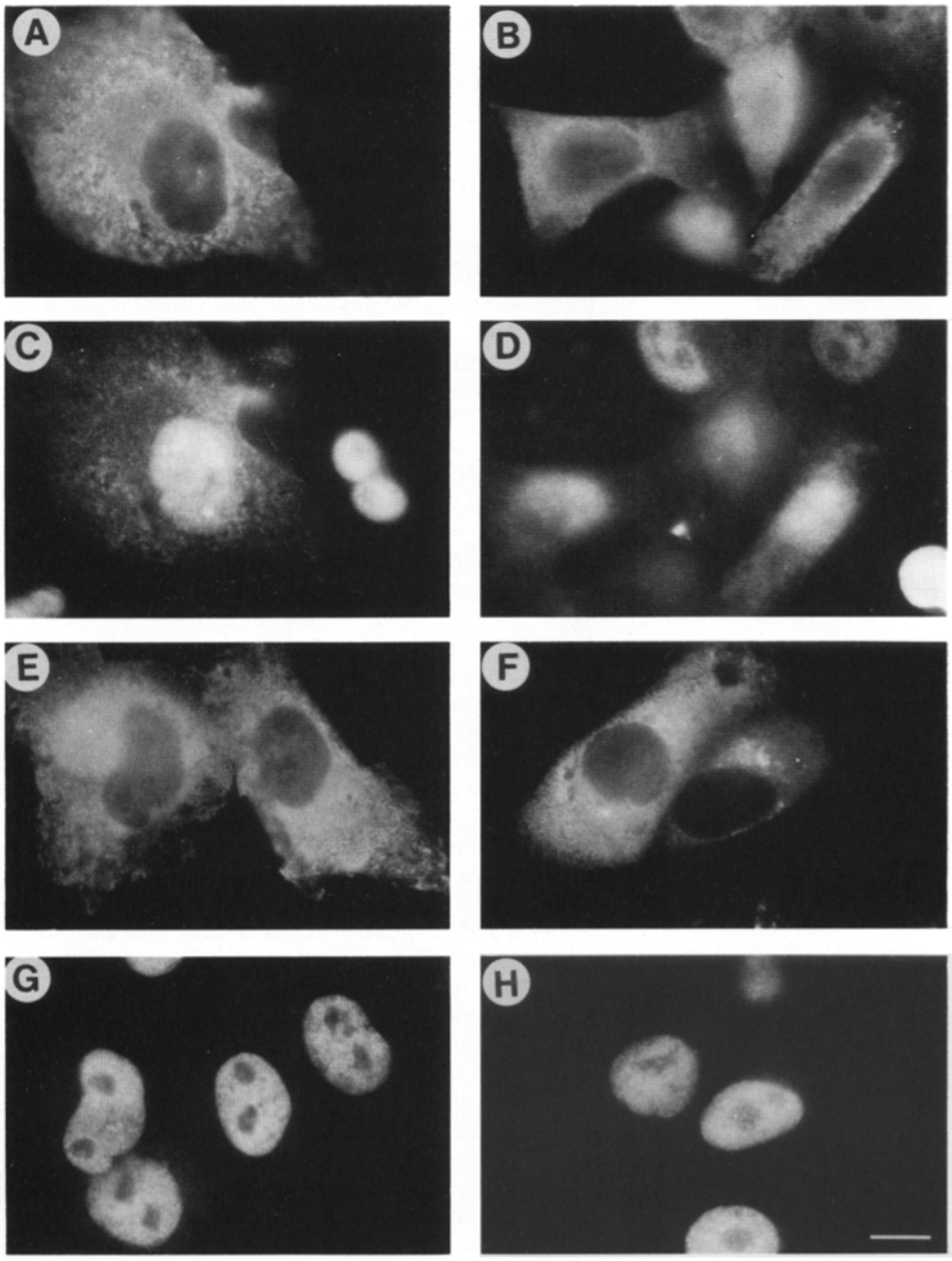

Figure 1. Subcellular localization of E1A in cells expressing T antigen mutants. Vero monkey kidney cells microinjected with plasmid DNA encoding FS (*A–D*), cT.3 (*E* and *G*), and cTFS (*F* and *H*) T antigens were superinfected with adenovirus 5, 1 h after microinjection. The cells were fixed 8 h later and stained for T antigen (*A, B, E, F*) and E1A (*C, D, G,* and *H*). T antigen was visualized with rabbit antiserum directed against T antigen followed by Texas red-conjugated second antibody. E1A protein was stained with the mAb m73 and an FITC-conjugated second antibody. Bar, 10 μm.

1988). The coupling ratio was estimated by gel electrophoresis to be between 15 and 20 peptides per carrier molecule. The ability of the NLS-bHSA conjugate to be transported to the nucleus was examined by microinjection of the peptide-albumin conjugate into the cytoplasm of Vero cells. NLS-bHSA was completely imported into the nucleus during a 15-min incubation at 37°C (data not shown). The behavior of NLS-bHSA is consistent with import behavior of similar NLS peptide conjugates reported by other groups (Lanford et al., 1986, 1990).

To test nuclear import of NLS-bHSA in the presence of FS T antigen, we employed a sequential microinjection assay. Vero cells were microinjected in the nucleus with plasmid DNA encoding FS T antigen, or as a control wild-type, cT.3, or cTFS T antigen. The cells were allowed to synthesize and accumulate FS T antigen at 37°C for 8 h. After this period, NLS-bHSA was microinjected into the cytoplasm of the same cells. The cells were incubated at 37°C for an additional hour and then prepared for double indirect immunofluorescent staining.

Wild-type T antigen did not impair the nuclear uptake of NLS-bHSA at any of the NLS-bHSA concentrations tested. In three independent experiments comprising over 100 cells positive for both wild-type T antigen and NLS-bHSA, NLS-bHSA was efficiently transported to the nucleus in 99% of the cells with no detectable residual cytoplasmic staining (Fig. 2, *A* and *C*). Similarly, cT.3 T antigen did not interfere with the nuclear import of NLS-bHSA (Fig. 2, *B* and *D*). In contrast, although NLS-bHSA did accumulate in the nucleus of cells expressing FS T antigen (Fig. 2, *E* and *F*), a significant cytoplasmic staining was also observed (Fig. 2, *I* and *J*). A complete nuclear distribution of NLS-bHSA was ob-

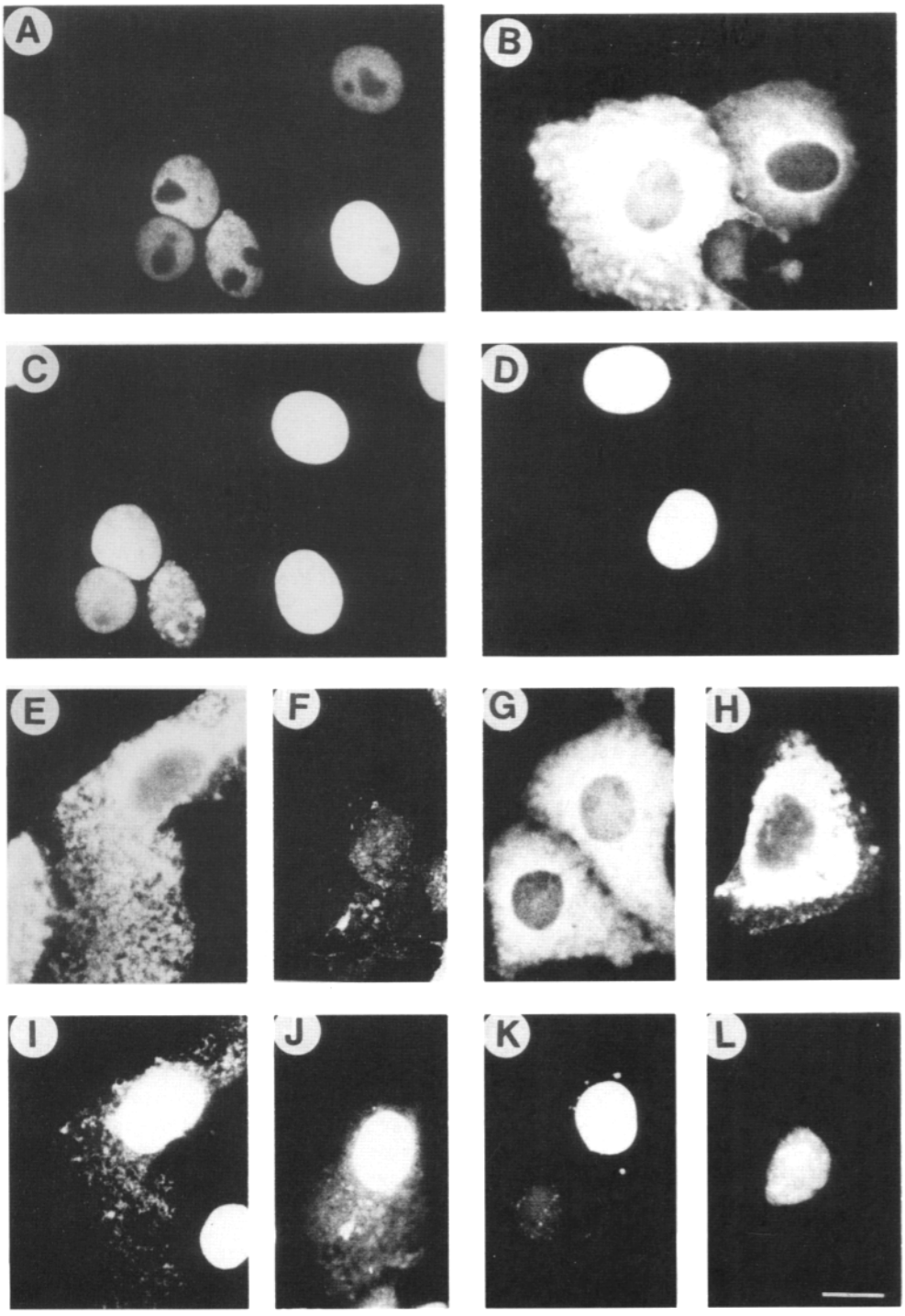

Figure 2. Nuclear uptake of NLS-bHSA is impaired in cells expressing FS T antigen. Vero cells were microinjected with plasmid DNA encoding wild-type (*A* and *C*), cT.3 (*B* and *D*), FS (*E*, *F*, *I*, and *J*) or cTFS (*G*, *H*, *K*, and *L*) T antigen. 8 h later, NLS-bHSA (shown here 1 mg/ml) was microinjected into the cytoplasm of the same cells. The nuclear transport of NLS-bHSA was allowed to proceed for 1 h before the cells were prepared for double immunofluorescent staining. The mAb Pab419 followed by an FITC-conjugated second antibody was used to visualize T antigen (*A*, *B*, *E–H*). Texas red-streptavidin was used to visualize the biotinylated NLS-bHSA (*C*, *D*, *I–L*). Bar, 10 μm.

served in only 25% of the cells expressing FS T antigen (20 out of 79 cells in three experiments); in the other 75% of the cells expressing FS T antigen (59 out of 79 cells in 3 experiments), NLS-bHSA was distributed in the nucleus and cytoplasm. The double mutant cTFS T antigen had little effect on the nuclear import of NLS-bHSA. When NLS-bHSA was injected into the cytoplasm of cells expressing cTFS T antigen (Fig. 2, *G* and *H*), the peptide-albumin conjugate accumulated in the nucleus with no residual cytoplasmic staining detectable in 85% of the cells (53 out of 62 cells in two experiments) (Fig. 2, *K* and *L*). In control experiments we have demonstrated that cells expressing FS T antigen 10–12 h after microinjection of plasmid DNA are viable and possess a nuclear envelope which functions as a selective barrier (data not shown). On the basis of these results, we conclude that FS T antigen interferes with the nuclear import of a variety of nuclear proteins in an NLS-dependent manner.

Receptor Titration Versus Pore Jamming

To explain the ability of FS T antigen to interfere with the nuclear transport apparatus with lethal consequences for the

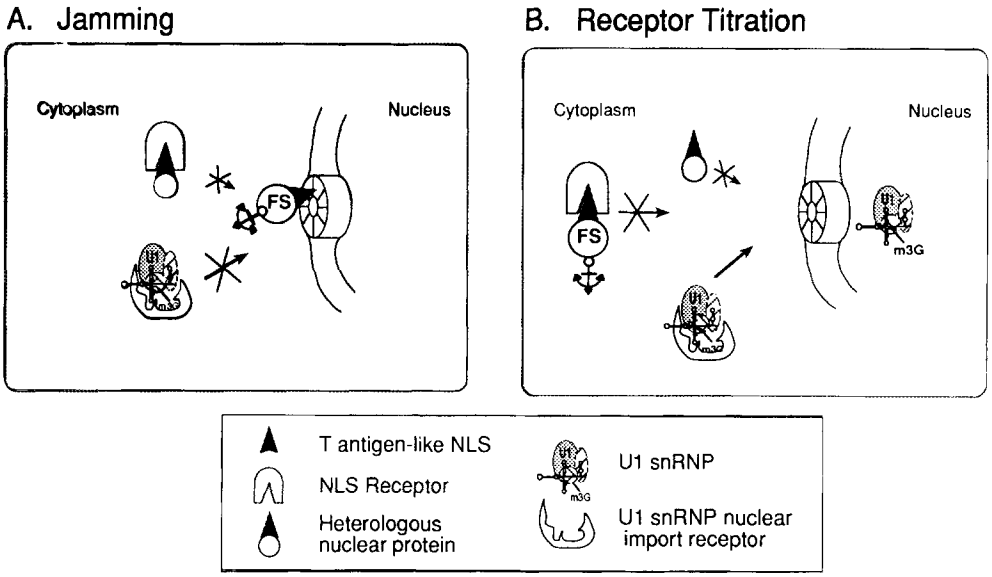

Figure 3. How does FS T antigen interfere with the nuclear transport machinery? Jamming and receptor titration models are described in the text.

cell, we have considered two models (Fig. 3). In the first model, which we have designated jamming (Fig. 3 *A*), FS T antigen engages the nuclear transport machinery and is transported to the NPCs. The subsequent translocation of FS T antigen is hindered by the novel carboxy terminal sequences. Jamming of the translocation machinery by FS T antigen would prevent other karyophilic molecules from being translocated through the NPC. In this model, transport through the NPC is limiting.

An alternative model (Fig. 3 *B*) is that the hydrophobic carboxy terminus of FS T antigen functions as a second intracellular targeting or cytoplasmic anchor sequence which competes with the NLS in determining the subcellular localization of the mutant protein. The NLS of cytoplasmically anchored FS T antigen would still be able to interact with soluble cellular factors which mediate nuclear import. FS T antigen could interfere with heterologous nuclear protein import by sequestering NLS receptors in an inactive complex in the cytoplasm, preventing the receptors from shuttling other proteins to the nucleus. The receptor titration model is based on the assumption that soluble cytoplasmic proteins present in a limited amount function as NLS receptors.

These models lead to several predictions for the behavior of the FS T antigen mutant that can be tested experimentally. If either of these models is correct, the mutant protein would most likely need to exceed a threshold concentration to interfere detectably with the nuclear transport of other karyophilic molecules. If cytoplasmically anchored FS T antigen were titrating out soluble NLS receptors, one would predict that FS T antigen would only interfere with the nuclear import of karyophilic molecules using the same class of import receptors. FS T antigen should not, however, interfere with the import of karyophilic molecules using different classes of transport receptors. Moreover, if FS T antigen were sequestering NLS receptors in the cytoplasm, one might expect purified microinjected FS T antigen to become anchored in the cytoplasm. In contrast, one would predict that if FS T antigen were jamming the NPC, purified FS T antigen would accumulate at the NPC after microinjection and it would interfere with multiple nuclear transport pathways.

Interference with Nuclear Protein Import Is Correlated with Accumulation of FS T Antigen

Both the jamming and receptor titration models (Fig. 3) predict that a threshold concentration of FS T antigen would be required to interfere with uptake of heterologous nuclear proteins. Indeed, the levels of FS T antigen used so far to detect the interference have been high (Figs. 1 and 2; van Zee et al., 1991; Schneider et al., 1988). To test whether low amounts of FS T antigen can also impair nuclear protein import, we again microinjected Vero cells with plasmid DNA encoding FS T antigen. After 2–10 h, we then microinjected the same cells a second time with NLS-bHSA conjugate. The cells were further incubated for 30 min at 37°C to allow nuclear uptake of NLS-bHSA. As a control, cells expressing WT T antigen were also microinjected with NLS-bHSA. The cells were then fixed and stained by double immunofluorescence and the subcellular location of NLS-bHSA in each doubly stained cell was evaluated.

Fig. 4 demonstrates that the low amounts of FS T antigen accumulated within 2 h after microinjection of SVFS DNA were unable to prevent efficient nuclear accumulation of NLS-bHSA (Fig. 4 *A*). At later times, as larger amounts of FS T antigen accumulated, nuclear uptake of NLS-bHSA was reduced; cytoplasmic NLS-bHSA was detectable in ~25% of the FS T antigen-expressing cells at 4 h and in ~90% at 10 h. In cells expressing WT T antigen, nuclear import of NLS-bHSA remained unaffected by accumulation of T antigen (Fig. 4 *B*). Nuclear import of NLS-bHSA was also unaffected in cells expressing cTFS T antigen (Fig. 2, *K* and *L*). Thus, high levels of FS T antigen are required to disrupt the nuclear protein import pathway, consistent with the predictions made for both the jamming and receptor titration models.

Nuclear Import of Digoxygenin-labeled U1 snRNA

To distinguish between the jamming and receptor titration models, we next examined the ability of FS T antigen to interfere with other nuclear transport pathways. It has been demonstrated that U1 snRNA is imported into the nucleus

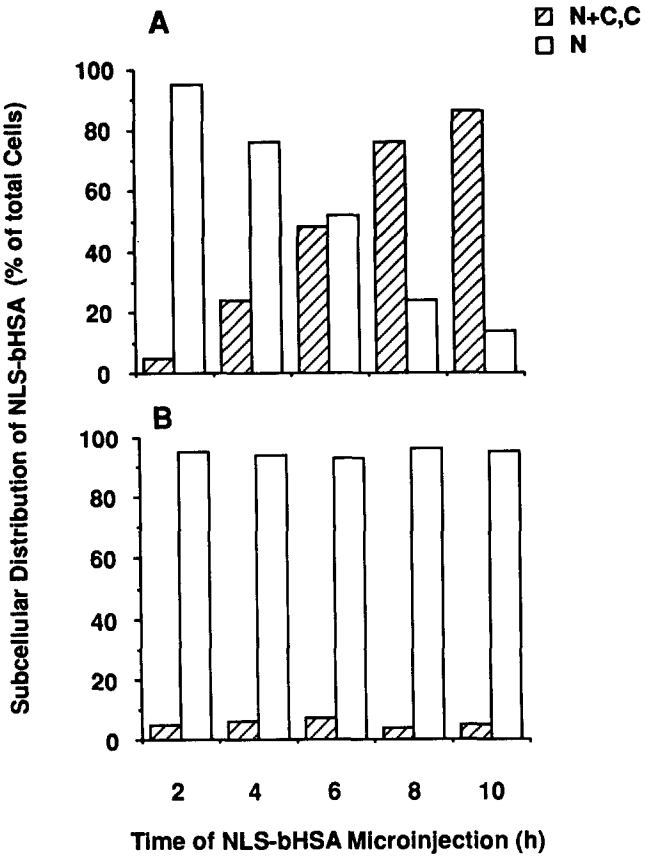

Figure 4. Disruption of NLS-bHSA nuclear import depends on accumulation of high levels of FS T antigen. Vero cells were microinjected with plasmid DNA (0.2 mg/ml) encoding FS (*A*) or WT (*B*) T antigen. After 2 to 10 h, NLS-bHSA (1 mg/ml) was microinjected into the cytoplasm of the same cells. Nuclear transport of NLS-bHSA was allowed to proceed for 30 min before the cells were processed for double immunofluorescent staining. The monoclonal antibodies Pab220 and Pab221, followed by Texas red-conjugated second antibody, were used to stain T antigen. FITC-streptavidin was used to visualize the biotinylated NLS-bHSA. The subcellular distribution of NLS-bHSA in cells expressing FS (*A*) or WT (*B*) T antigen was evaluated quantitatively. The percentage of cells with NLS-bHSA in the nucleus only is indicated by unshaded columns. The percentage with NLS-bHSA in the cytoplasm or nucleus and cytoplasm is indicated by shaded columns. The results from 37 to 85 FS-positive cells and 62 to 116 WT-positive cells at each time point are depicted. Qualitatively similar results were obtained using a lower concentration of NLS-bHSA (0.5 mg/ml).

in *Xenopus* oocytes via a transport pathway distinct from that used by proteins targeted by the T antigen or nucleoplasmin NLS (Michaud and Goldfarb, 1991, 1992; Fischer et al., 1991). Specifically, more recent competition studies in *Xenopus* oocytes carried out with in vitro reconstituted snRNPs show that the Sm core domain of U1 snRNPs contains a nuclear location signal that acts independently of the m_3G cap and is not the same as the NLSs that target karyophilic proteins to the nucleus (U. Fischer, V. Sumpter, R. Lührmann, manuscript in preparation). We, therefore, wished to test whether FS T antigen interferes with the nuclear import of U1 snRNAs in somatic cells. As an alternative to radiolabeled U1 snRNA, we synthesized U1 snRNA in vitro from a *Xenopus laevis* U1 snRNA gene template in the presence of digoxygenin-labeled UTP and an m^7GpppG dinucleotide cap analog (as described in Materials and Methods).

To determine whether this *Xenopus* digoxygenin-labeled U1 snRNA (DIG-U1 snRNA) would be recognized by the nuclear import machinery in somatic monkey tissue culture cells, the subcellular localization of DIG-U1 snRNA was determined by indirect immunofluorescent staining at different times after microinjection into the cytoplasm of Vero cells. The kinetics of DIG-U1 snRNA nuclear uptake are shown in Fig. 5, *A* to *E*. Whereas NLS-bHSA is transported to the nucleus within 15–30 min at 37°C, a significant nuclear accumulation of DIG-U1 snRNA is first detectable at 2 h after microinjection, and 6 h are required for virtually all of the DIG-U1 snRNA to be transported to the nucleus. The slow import kinetics of U1 snRNA most likely reflect the time required for assembly and processing of U1 snRNA into mature snRNPs. In a second study it has been verified by mutational analysis that the nuclear transport of U1 RNA in somatic cells is a mediated process and requires the Sm core domain (U. Fischer, K. van Zee, J. Biele, I. Mattaj, E. Fanning, R. Lührmann, manuscript in preparation). Moreover, nuclear uptake of DIG U1 snRNA was inhibited by anti-Sm antibodies microinjected into the cytoplasm, but not by nonspecific antibodies (U. Fischer, J. Biele, R. Lührmann, manuscript in preparation). The import kinetics of DIG-U1 snRNA, the requirement for the Sm core domain for nuclear import, and the inhibition of nuclear uptake by anti-Sm antibody argue strongly that DIG-U1 snRNA transport in microinjected cells is preceded by U1 snRNP assembly.

It is unlikely that the nuclear import of DIG-U1 snRNA results from a nonspecific interaction of digoxygenin with the nuclear transport machinery as a digoxygenin-labeled tRNA was not transported to the nucleus following microinjection into the cytoplasm (data not shown). Furthermore, in *Xenopus* oocytes the nuclear import behavior of digoxygenin-labeled U snRNAs is indistinguishable from that of radiolabeled U snRNAs in *Xenopus* oocytes (U. Fischer and R. Lührmann, unpublished data).

FS T Antigen Does Not Interfere with the Nuclear Import of DIG-U1 snRNA

The specific nuclear import of *Xenopus* DIG-U1 snRNA suggests that it is properly assembled into U snRNP complexes in monkey cells and is an appropriate import substrate to use in determining the influence of FS T antigen on the transport of other classes of karyophilic molecules. Next, Vero cells were microinjected with plasmid DNA encoding FS, WT, cT.3, or cTFS T antigen. The cells were incubated at 37°C for 6 h before DIG-U1 snRNA was microinjected into the cytoplasm of the same cells. After microinjection of DIG-U1 snRNA into the cytoplasm, the cells were incubated for an additional 6 h at 37°C to allow U1 snRNPs to be assembled and transported to the nucleus. After fixation, the subcellular distribution of DIG-U1 snRNA in the presence of the different T antigen species was examined by double indirect immunofluorescent staining. In all cells expressing FS T antigen (78 cells in three experiments) (Fig. 6 C), DIG-U1 snRNA accumulated efficiently in the nucleus (Fig. 6 *G*). No residual cytoplasmic staining of DIG-U1 snRNA was visible.

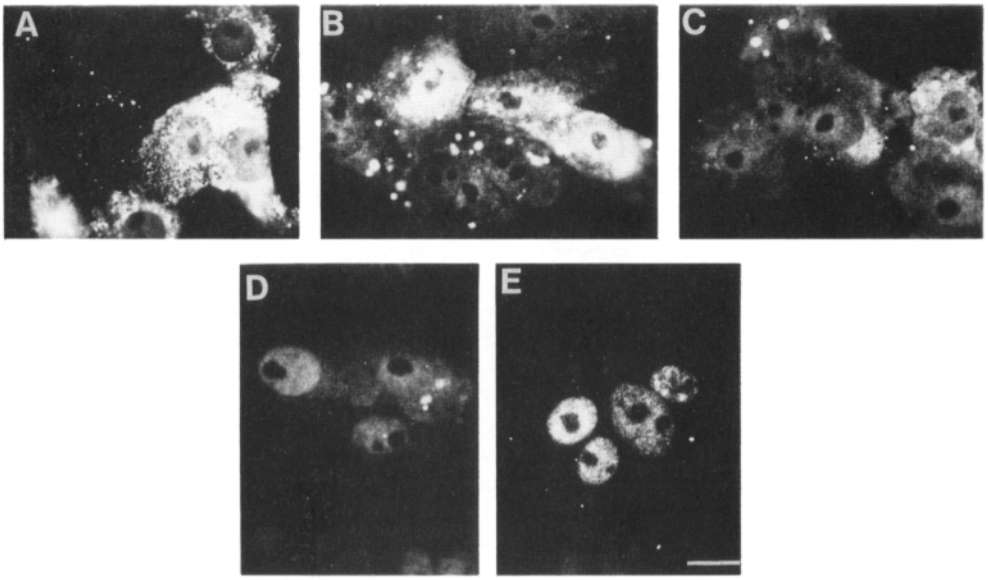

Figure 5. Digoxygenin-labeled U1 snRNA is imported into the nucleus after microinjection into the cytoplasm of Vero cells. Digoxygenin-labeled U1 snRNA (*A–E*) were microinjected into the cytoplasm of Vero cells. The cells were prepared for immunofluorescent staining 30 min (*A*), 2 h (*B*), 4 h (*C*), 6 h (*D*), and 8 h (*E*) after microinjection. The subcellular distribution of digoxygenin-labeled RNAs was detected using a monoclonal antibody from mouse against digoxygenin (Boehringer Mannheim) and an FITC-conjugated second antibody. Bar, 10 μm.

Similarly, DIG-U1 snRNA (Fig. 6, *E*, *F*, and *H*) accumulated efficiently in the nucleus in the presence of wild-type, cT.3, and cTFS T antigens (Fig. 6, *A*, *B*, and *D*, respectively). Furthermore, as the subcellular distribution of DIG-U1 snRNA in the presence of FS, wild-type, cT.3, or cTFS T antigen at an earlier time after the injection of DIG-U1 snRNA was comparable, we conclude that the import kinetics of U1 snRNA are not altered by the presence of FS T antigen. Together these results suggest that FS T antigen does not interfere with the nuclear import pathway of U1 snRNA.

FS T Antigen Does Not Accumulate at the NPC

According to the jamming model (Fig. 3 A), an enrichment of FS T antigen at the NPC would be expected if FS T antigen were plugging the NPC. To examine whether FS T antigen accumulates at the NPC, we monitored the subcellular distribution of the mutant protein following microinjection of purified protein into the cytoplasm of HeLa cells. Recombinant FS, wild-type, and cT.3 T antigens (designated bvFS, bvWT, and bvcT.3, respectively) were immunoaffinity purified from baculovirus-infected insect cells as described in Materials and Methods. The integrity and purity of the recombinant proteins were determined by gel electrophoresis followed by silver staining (Fig. 7 *A*). Each of the purified proteins was microinjected into the cytoplasm of HeLa cells, and the cells were incubated at 37°C for 1 h before being fixed for indirect immunofluorescent staining. bvWT T antigen was rapidly transported to the nucleus, resulting in a complete nuclear localization of the protein within the 1 h incubation (Fig. 7 *B*). Consistent with its defective NLS, bvcT.3 T antigen failed to accumulate to significant levels in the nu-

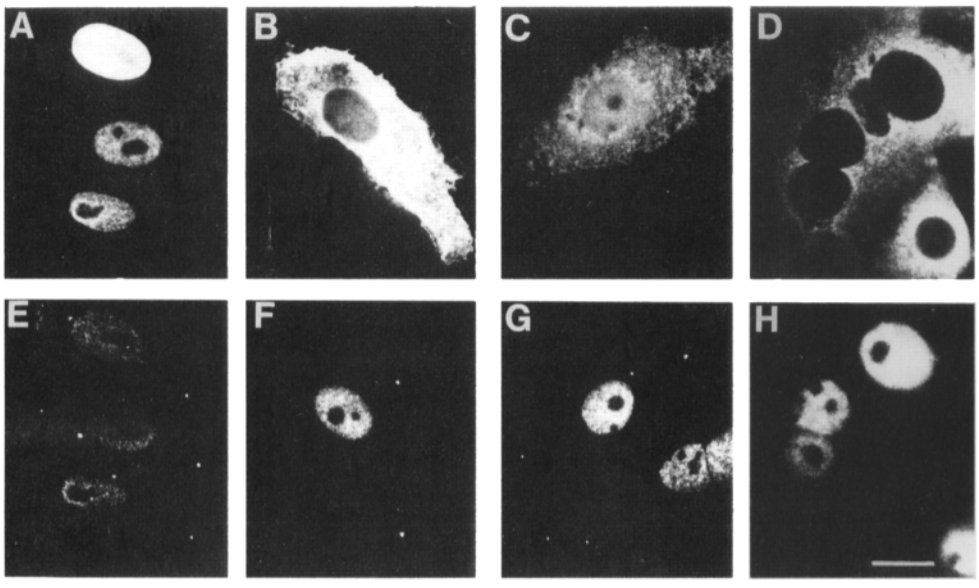

Figure 6. FS T antigen does not interfere with the nuclear import of DIG-U1 snRNAs. Vero cells were microinjected in the nucleus with plasmid DNA encoding wild-type (*A* and *E*), cT.3 (*B* and *F*), FS (*C* and *G*), or cTFS (*D* and *H*) T antigen. 6 h later, the same cells were microinjected in the cytoplasm with DIG-U1 snRNA. The cells were incubated for an additional 6 h at 37°C and then prepared for double indirect immunofluorescent staining. A rabbit antiserum directed against T antigen and a Texas red-conjugated second antibody were used to visualize T antigen (*A–D*). Mouse antidigoxygenin and an FITC-conjugated second antibody were used to visualize DIG-U1 snRNA (*E–H*). Bar, 10 μm.

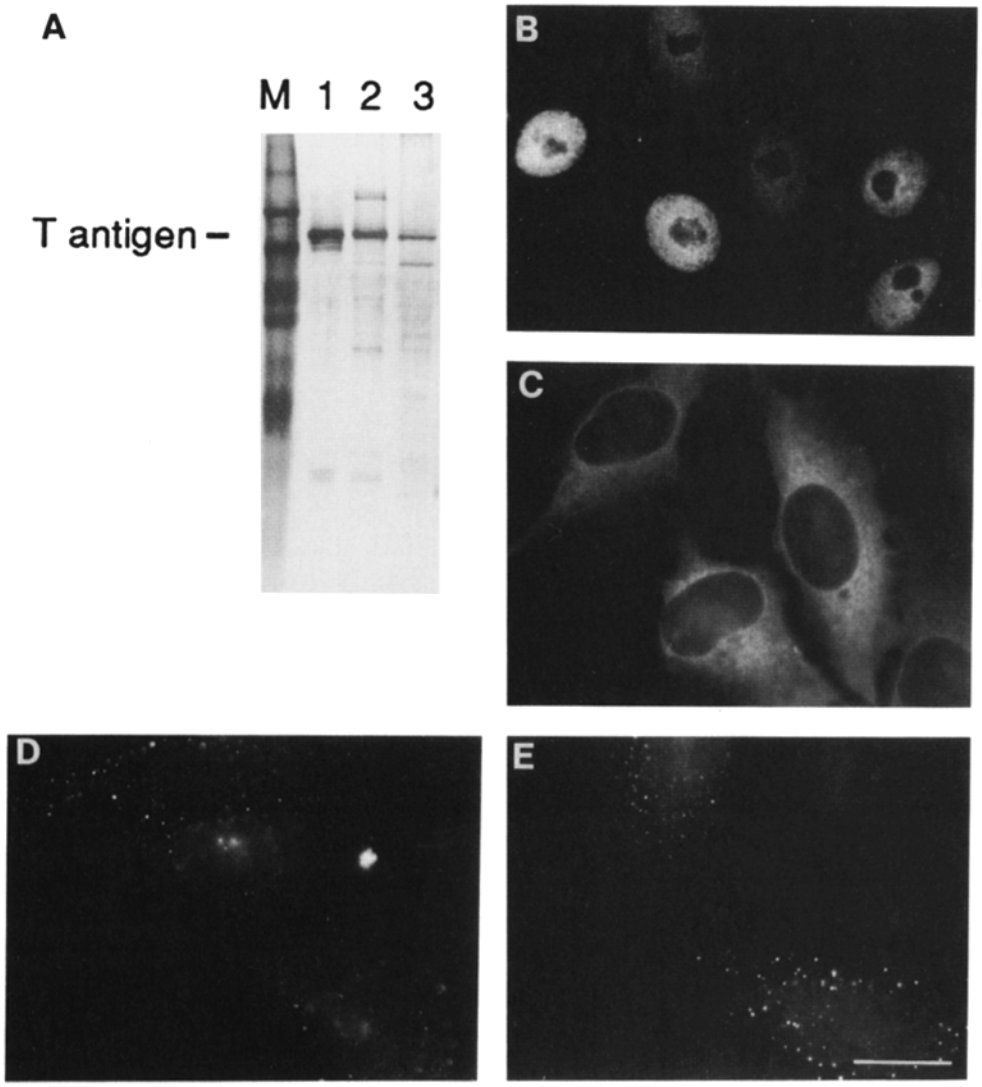

Figure 7. Purified FS T antigen protein does not accumulate in a perinuclear region after microinjection into the cytoplasm of HeLa cells. (*A*) bvWT (lane *1*), bvCT.3 (lane *2*), and bvFS (lane *3*) T antigens were purified from insect cells infected with the appropriate recombinant baculovirus as described in Materials and Methods and analyzed by gel electrophoresis (Laemmli, 1970) followed by silver staining (Heukeshoven and Dernick, 1988). 1 μg of bvWT and bvcT.3 (lanes *1* and *2*, respectively) and 0.5 μg of bvFS (lane *3*) T antigens were loaded in each lane. Prestained marker proteins (*M*) (α2 macroglobulin, 180,000; β-galactosidase, 116,000; fructose-6-phosphate kinase, 84,000; pyruvate kinase, 58,000; fumerase, 48,500; lactic dehydrogenase, 36,500; triose phosphate isomerase, 29,700 D) were purchased from Sigma Chemical Co. T antigen is indicated by a bar. In lane *3* a minor band migrating faster than FS T antigen is likely to represent a breakdown product of FS T antigen. The subcellular distribution of bvWT (*B*), bvcT.3 (*C*), and bvFS (*D* and *E*) was determined by indirect immunofluorescent staining 1 h after microinjection of the proteins into the cytoplasm of HeLa cells. Proteins were injected at a concentration of 1 mg/ml (bvWT and bvcT.3) or 0.8 mg/ml (bvFS). Cells were stained with Pab419 followed by an FITC-conjugated second antibody. Bar, 10 μm.

cleus in any of the injected cells (Fig. 7 *C*). The subcellular distribution of bvFS T antigen 1 h after microinjection into the cytoplasm is shown in Fig. 7, *D* and *E*. We observed a punctate cytoplasmic localization of bvFS T antigen and did not observe a distinct perinuclear rim staining characteristic of proteins localized at the nuclear envelope. This punctate distribution of FS T antigen resembled the staining pattern we observed in HeLa cells expressing FS T antigen from microinjected SVFS DNA (data not shown) and confirm our previous results that FS T antigen is anchored in the cytoplasm (van Zee et al., 1991).

Discussion

In this study, we have examined the ability of a cytoplasmically anchored nuclear protein, FS T antigen, to disrupt nuclear transport pathways. FS T antigen impairs the nuclear import of several heterologous proteins, including adenovirus fiber, E1A, and DNA binding proteins, and a synthetic NLS peptide-albumin conjugate (Schneider et al., 1988; van Zee et al., 1991; Figs. 1 and 2; data not shown). Interference with nuclear import of each of these proteins depends on the integrity of the NLS in the FS T antigen, as inactivation of the NLS in the double mutant dTFS T antigen relieved the interference with their import. These results suggest that FS T antigen initially engages the nuclear transport machinery in a signal-dependent manner, but then inactivates or sequesters at least one component of the import pathway utilized by a wide variety of heterologous proteins, thereby blocking or retarding nuclear protein import.

How does FS T antigen interfere with nuclear protein transport? Two simple models, shown in Fig. 3, focus on two components of the transport pathway thought to be utilized by most nuclear proteins, nuclear pores and NLS receptor

proteins. Either of these models, termed pore jamming and receptor titration, would account for the NLS-dependent disruption of nuclear protein import by FS T antigen. In the jamming model, FS T antigen requires a functional NLS to reach the NPC, where its translocation and that of other karyophilic molecules into the nucleus would be retarded by the hydrophobic COOH terminus of FS T antigen. In the receptor titration model, FS T antigen is anchored in the cytoplasm through its COOH terminus, but requires the NLS to interact with and sequester NLS-binding factors required for nuclear import. An important prediction of both models is that disruption of nuclear protein import should be proportional to the concentration of FS T antigen in the cell. Consistent with this prediction, nuclear import of a synthetic peptide NLS-albumin conjugate was impaired only when large amounts of FS T antigen had accumulated.

One important distinction between the pore jamming and the receptor titration models lies in whether FS T antigen is able to disrupt multiple nuclear import pathways mediated by different classes of karyophilic signal receptors. Kinetic experiments in *Xenopus* oocytes have shown that while certain nuclear proteins, for example T antigen and nucleoplasmin, compete among themselves for NLS receptors, U1 and U2 snRNPs do not compete with these nuclear proteins for the same NLS receptor (Michaud and Goldfarb, 1991, 1992).

In our experiments in somatic cells, we assumed that the labeled U1 snRNA injected into the cytoplasm would become properly assembled into U1 snRNPs before nuclear transport as it does in oocytes. Recent studies of nuclear import of U1 RNA in somatic cells confirm that it is a signal-mediated process and depends on the integrity of the Sm core domain (U. Fischer, K. van Zee, J. Biede, I. Mattaj, E. Fanning, R. Lührmann, manuscript in preparation). Additional evidence supporting the idea that nuclear import of U1 snRNA in somatic cells is preceded by snRNP assembly comes from the observation that anti-Sm antibodies microinjected into the cytoplasm inhibit U1 snRNA import, while nonspecific antibodies have no effect (U. Fischer, J. Biele, R. Lührmann, manuscript in preparation). Thus, it seems likely that nuclear import of U1 snRNA in somatic cells is mediated by karyophilic signal receptors specific for U1 snRNPs.

According to the pore jamming model, we expected FS T antigen to interfere with nuclear transport of U1 snRNPs. Moreover, if translocation of FS T antigen through the NPCs were retarded, we expected that purified FS T antigen introduced into the cytoplasm by microinjection should be enriched at the NPCs. As demonstrated in Figs. 6 and 7, our data do not fulfill either of these predictions. However, our data are consistent with the receptor titration model.

The receptor titration model is based on the assumption that soluble cytoplasmic factors that are present in limited amounts in the cytoplasm function as NLS receptors. There is substantial evidence from both mammalian and *Xenopus* in vitro nuclear import assays to support the existence of soluble cytoplasmic NLS receptors (Adam et al., 1990; Adam and Gerace, 1991; Breeuwer and Goldfarb, 1990; Newmeyer and Forbes, 1988, 1990; Moore and Blobel, 1992). Newmeyer and Forbes (1990) have demonstrated that two cytosolic factors which are sensitive to the sulfhydryl alkylating reagent N-ethylmaleimide, NIF-1 and NIF-2, participate in the import of proteins into purified rat liver nuclei healed in *Xenopus* egg extracts. Cytosolic factors are also required for at least two different steps of nuclear protein import into digitonin-permeabilized cultured mammalian cells (Adam et al., 1990; Moore and Blobel, 1992). One of these factors binds to NLSs and constitutes a functional NLS receptor (Adam and Gerace, 1991). More recently, a novel cytosolic factor which interacts directly with O-linked glycoproteins of the NPC has been shown to be required for nuclear import in vitro (Sterne-Marr et al., 1992). Sequestration of any of these putative NLS receptors by FS T antigen could impair nuclear protein import.

The role of cytosolic NLS receptors remains controversial, however, in light of the fact that several groups have not found a requirement for cytosolic extracts in their in vitro nuclear transport assays (Markland et al., 1987; Imamoto-Sonobe et al., 1988; Kalinich and Douglas, 1989; Silver et al., 1989; Parniak and Kennady, 1990). One possible explanation for the discrepancy in the requirement for cytosolic extracts is that an NLS receptor which shuttles between the nucleus and cytoplasm may mediate nuclear protein import. Sufficient quantities of a shuttling receptor could be isolated with the nuclei under some conditions to obviate a requirement for cytosolic factors in in vitro nuclear transport assays. The identification of a rat NLS-binding protein which shuttles between the nucleolus and cytoplasm (Meier and Blobel, 1990, 1992) and the observation that some NLS-binding proteins are present in both cytosolic and nuclear extracts (e.g., Adam et al., 1989) has lent support to the possibility that nuclear import receptors may escort their cargo into the nucleus and then shuttle back to the cytoplasm to mediate further rounds of protein targeting. The receptor titration model does not exclude the possibility that FS T antigen could also sequester a shuttling NLS-receptor in the cytoplasm and prevent it from escorting other proteins back into the nucleus.

In summary, the cytoplasmic anchoring of FS T antigen, the ability of FS T antigen to interfere in an NLS- and concentration-dependent manner with nuclear transport of a variety of nuclear proteins, and its inability to block nuclear uptake of U1 snRNAs lead us to favor the receptor titration model. Although the jamming model is not completely ruled out by the available data, it seems unlikely to us in light of the ability of FS T antigen to impair the nuclear import of only certain classes of karyophilic molecules. If cytoplasmic NLS receptors are in fact sequestered by cytoplasmically anchored FS T antigen, as proposed by the receptor titration model, one would predict that nuclear protein import could be restored by supplying excess NLS receptor. Thus, we anticipate that FS T antigen may be a useful tool to study the in vivo function of nuclear protein NLS receptors.

We are grateful to Iain Mattaj, European Molecular Biology Laboratory, Heidelberg, for providing U1 snRNA plasmids. We would also like to thank Wolfgang Deppert, Heinrich-Pette-Institut, Hamburg, for providing the polyclonal antisera to T antigen, Dr. H. J. Gross, University of Würzburg, Germany, for providing a tRNA plasmid, Akis Zorbas, Institut für Biochemie, Munich, for adenovirus 5, and Ismail Moarefi and Avril Arthur for help with figures. Also, many thanks to all members of the Fanning laboratory for their moral support and advice.

This work was supported by the Deutsche Forschungsgemeinschaft (Fa138/4-2), Bundesministerium für Forschung und Technologie (Genzentrum), and Fonds der Chemischen Industrie and is a partial fulfillment of the requirements for the doctoral degrees of K. van Zee and A. Dickmanns.

Received for publication 6 July 1992 and in revised form 10 December 1992.

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