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Nuclear transport kinetics depend on phosphorylation-site-containing sequences flanking the karyophilic signal of the Simian virus 40 T-antigen

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Selective nuclear protein transport was analyzed in single living cells. Hybrid proteins consisting of short stretches of the Simian virus 40 T-antigen and of the almost complete β-galactosidase moiety were generated by molecular genetic methods and injected into the cytoplasm of rodent hepatoma cells. A histochemical assay showed that all proteins containing the karyophilic signal of the T-antigen (residues 126/127 – 132) were equally well accumulated by the nucleus within 15 h after injection. Microfluorimetric measurements of nuclear transport kinetics, however, revealed large differences. Proteins containing the karyophilic signal without flanking sequences were taken up by the nucleus on a time scale of hours. The same held for a protein containing T-antigen residues 127 – 147. However, a protein containing T-antigen residues 111 – 135 was accumulated by the nucleus with a half-time of 8 – 10 min reaching an equilibrium nucleocytoplasmic concentration ratio of ≥15. Photobleaching measurements showed that, independently of subcellular localization, the mobility of all proteins was quite large. Thus, our measurements revealed a striking effect of T-antigen residues 111 – 125 on the kinetics of nuclear transport. Residues 111 – 125 do not seem to contain a second karyophilic signal. Conspicuously, however, they comprise a cluster of phosphorylation sites.

Key words: nucleus/phosphorylation/photobleaching/T-antigen/transport

Introduction

Nucleocytoplasmic transport functionally links genomic information with protein biosynthesis and for this reason assumes a crucial position in the cellular machinery. Nevertheless, the bulk of mass exchange between nucleus and cytoplasm is based on an energetically favorable but rather coarse mechanism. The nuclear envelope has properties of a highly permeable molecular sieve with an effective pore radius of ~50 Å (Paine *et al.*, 1975; Peters, 1984). Thus, many small and intermediately sized molecules are distributed between cytoplasm and nucleus by diffusion. A quantitatively smaller but highly significant fraction of nucleocytoplasmic transport processes concerns molecules which exceed in size the effective pore radius. This led to the suggestion (De Robertis *et al.*, 1978) that certain large nuclear proteins contain a 'signal' which somehow directs their intracellular transport. The molecular nature of karyophilic signals could be elucidated only recently by molecular genetic methods. In 1984 it was shown for one viral (Kalderon *et al.*, 1984a) and two different yeast proteins (Hall *et al.*, 1984; Silver *et al.*, 1984) that their nuclear localization is caused by short stretches of amino acid residues which are an integral part of the mature protein not removed during nuclear transport. In the meantime karyophilic sequences have been identified in several proteins [for reviews see Dingwall and Laskey (1986); Peters (1986); Newport and Forbes (1987) and Gerace and Burke (1988)].

In general, karyophilic sequences have been identified and characterized by their ability to affect the subcellular equilibrium distribution of carrier proteins. In their exemplary studies Smith and colleagues constructed a battery of plasmids containing mutant genes of the Simian virus 40 (SV40) T-antigen (Kalderon *et al.*, 1984a,b; Kalderon and Smith, 1984). The plasmids were injected into the nucleus of cultured mammalian cells. The cells were fixed and the intracellular localization of expression products was assessed by indirect immunofluorescence 16 – 18 h after injection. By these means it was shown that the karyophilic sequence of the SV40 T-antigen comprises residues 126 or 127 to 132 or 133 and thus consists of the six – eight predominantly basic amino acid residues PKKKRKV (Kalderon *et al.*, 1984a,b; Kalderon and Smith, 1984). Furthermore, it was concluded that the karyophilic sequence is an autonomous entity which exerts its function independently of flanking sequences (Kalderon and Smith, 1984) in a variety of molecular environments (Kalderon *et al.*, 1984b; Colledge *et al.*, 1986). Particular attention was paid to the phosphorylation sites which have been previously identified (Scheidtmann *et al.*, 1982) in the region flanking the karyophilic sequence on its N-terminal border. Mutation of these sites (S^{106}, S^{111}, S^{112}, S^{123} and T^{124}) did not affect nuclear localization in the assay used by Kalderon and Smith (1984) which seemed to devaluate speculations (Lanford and Butel, 1980; Scheidtmann *et al.*, 1984b) on correlations between phosphorylation state and nuclear transport.

The present study employed analytical fluorescence microscopy to directly monitor the kinetics of nuclear transport in single living cells injected with engineered proteins. By these means the basic role of the karyophilic sequence of the SV40 T-antigen was confirmed. Residues 126 – 132 were necessary and sufficient to localize large proteins to the nucleus. We discovered, however, that residues 111 – 125 which flank the karyophilic sequence on its N-terminal border have a rather dramatic effect on the kinetics of nuclear transport. This region of the T-antigen does not contain basic residues or any other configuration homologous to previously defined karyophilic sequences. The region is distinguished, however, by phosphorylation sites. This suggests a reconsideration of the participation of phosphorylation in nuclear transport.

Results

The hybrid proteins

As indicated schematically in Figure 1 the hybrid proteins P1−P4 employed in the present study all consisted of a first linker, a fragment of the SV40 T-antigen, a second linker, and the β-galactosidase moiety. The primary structure of the constructs is given in Materials and methods. For all of the hybrid proteins except P2 two variants were constructed with either a lysine (K-variant) or a threonine (T-variant) residue in position 128. The hybrid proteins could be expressed in *Escherichia coli* in high yields and were purified by affinity chromatography. Purification made use of the β-galactosidase activity of the hybrid proteins. This implies that the isolated proteins occurred as tetramers. The molecular mass of monomeric subunits was estimated from the primary structure to be 117−120 kd depending on the size of T-antigen fragments and linkers. As the fluorescent label we chose 5-iodoacetamidofluorescein (IAF) because it reacts with sulfhydril groups and not with lysine residues which are, for instance, part of the karyophilic signal sequence. The labeling ratio was kept small (0.5−1 mol IAF/mol subunit).

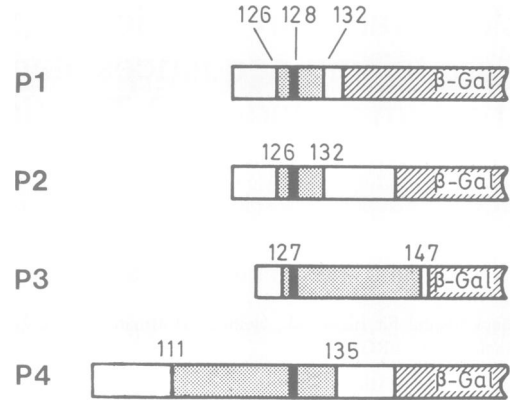

Fig. 1. Scheme of the hybrid proteins constructed in the present study. The N- and C-terminal amino acid residues of the SV40 T-antigen fragments are indicated by numbers. β-Gal, β-galactosidase moiety. Empty boxes are linker regions. For each of the proteins except P2 a wild-type variant carrying a lysine residue in position 128 and a cytoplasmic variant with a threonine in position 128 was constructed.

The cells

In all experiments we used polykaryons obtained by treating confluent monolayers of rat hepatoma tissue culture (HTC) cells with polyethylene glycol (PEG). We have previously characterized intracellular transport in HTC polykaryons with regard to molecular mobility (Lang *et al.*, 1986), passive permeability of the nuclear envelope (Lang *et al.*, 1986) and specific nuclear protein transport (Schulz and Peters, 1986, 1987; Dabauvalle *et al.*, 1988). Initially, polykaryons were used for technical reasons (Lang *et al.*, 1986). In the present context polykaryons have the advantage not to undergo mitosis, a process which could profoundly influence the intracellular distribution of macromolecules because the nuclear envelope is dis- and re-assembled.

Histochemical assay of intracellular equilibrium distribution

A histochemical assay which makes use of the β-galactosidase activity of the hybrid proteins P1−P4 generating an intensive blue stain from the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was employed. Several polykaryons were injected at a time. The culture dish with injected cells was then kept in the incubator and processed later for the X-gal reaction.

The X-gal assay showed that 15 h after cytoplasmic injection all lysine variants of the hybrid proteins were exclusively localized in the nucleus. All threonine variants, however, as well as pure β-galactosidase, remained exclusively or at least predominantly in the cytoplasm. Indeed, the subcellular distribution of all K-variants, on the one hand, and of all T-variants and pure β-galactosidase, on the other hand, looked alike in this assay. An example pertaining to P1 and P4 is given in Figure 2.

The X-gal assay also showed that the hybrid proteins preserved their β-galactosidase activity after injection into the cytoplasm and transport into the nucleus. This implies that the proteins occurred as tetramers of ~480 kd. This is consistent with the size limit for nuclear protein transport as determined by Lanford *et al.* (1986) who observed that

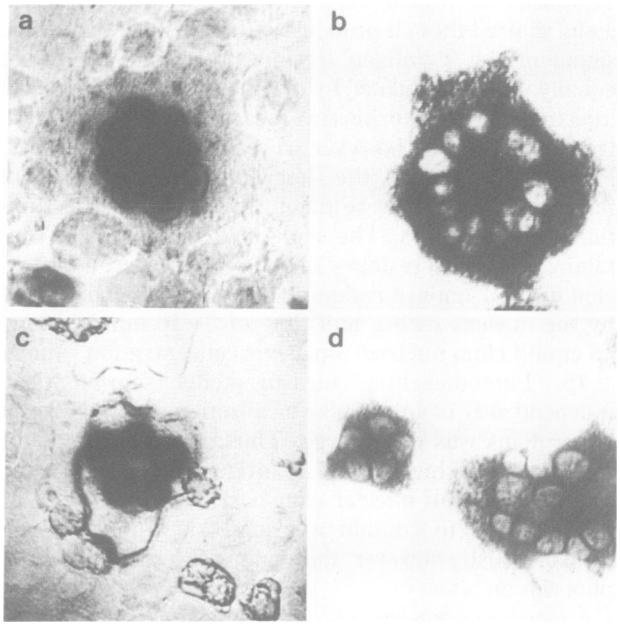

Fig. 2. Histochemical assay of equilibrium subcellular distribution. Hybrid proteins were injected into the cytoplasm of HTC polykaryons. Fifteen hours after injection the intracellular distribution of hybrid proteins was assayed by the X-gal method. (**a**) Protein P1K, (**b**) P1T, (**c**) P4K, (**d**) P4T.

ferritin (465 kd) but not IgM (970 kd) could be targeted to the nucleus by conjugation with a synethic signal peptide.

Microfluorimetric measurements of nuclear transport kinetics

The kinetics of nucleo-cytoplasmic transport were determined by means of the fluorescence microphotolysis (photobleaching) apparatus. Upon injection of fluorescently labeled protein into the cytoplasm the fluorescence of small areas in the nucleus and a cytoplasmic area close to the nucleus was measured as a function of time after cytoplasmic injection. From these measurements the time-dependence of the nucleo-cytoplasmic fluorescence ratio *Fn/c* was derived. As discussed previously (Lang *et al.*, 1986) at zero time *Fn/c*

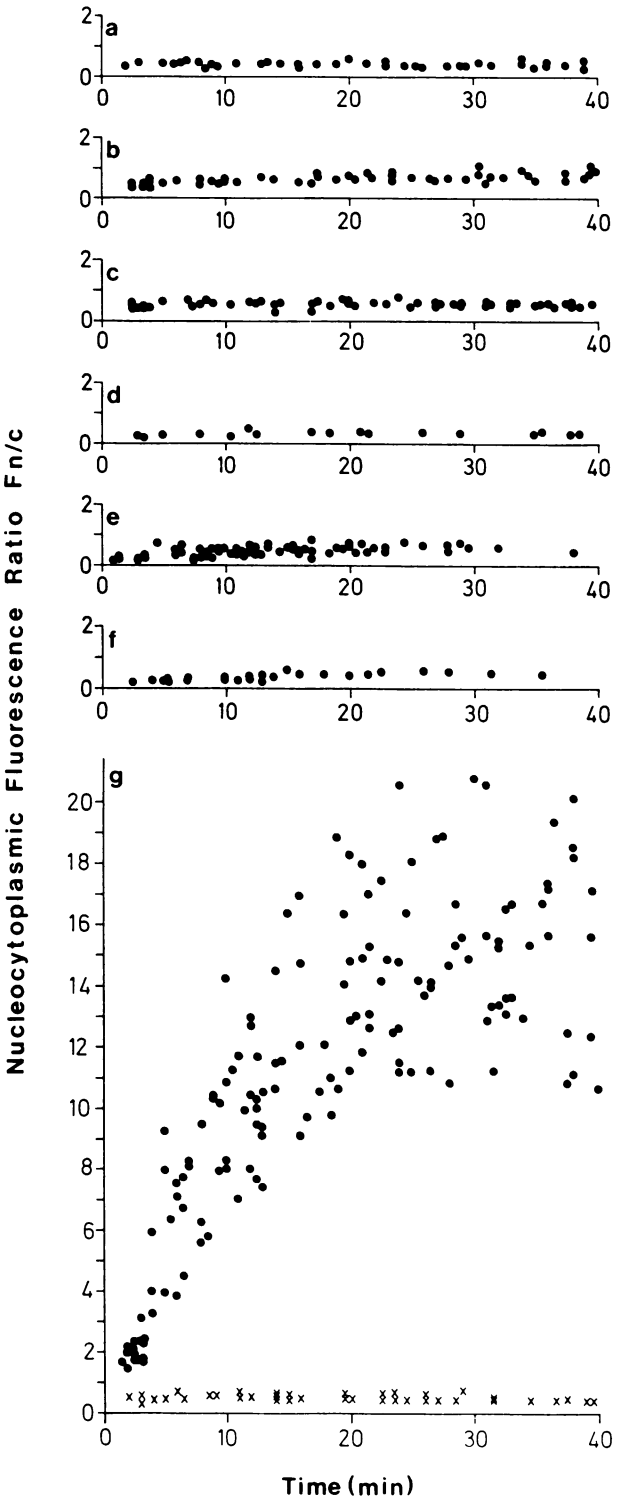

Fig. 3. Kinetics of nuclear transport as measured by laser microfluorimetry. Fluorescently labeled hybrid proteins were injected into the cytoplasm of HTC polykaryons. The nucleocytoplasmic fluorescence ratio *Fn/c* was determined by microfluorimetry and plotted versus time after injection. (**a**) Pure β-galactosidase, (**b**) P1K, (**c**) P1T, (**d**) P2K, (**e**) P3K, (**f**) P3T, (**g**) P4K (dots) and P4T (crosses).

is not zero but ~0.4 because fluorescence originating from cytoplasmic layers above and below the nucleus is contained in the measurement of 'nuclear' fluorescence. For the same reason the *Fn/c* ratio is an approximate but not a direct measure of the nucleocytoplasmic concentration ratio *Cn/c* (Schulz and Peters, 1987); in our experimental conditions the *Fn/c* ratio sets the lower limit for the *Cn/c* ratio.

A base line for nucleocytoplasmic transport of the hybrid proteins was provided by measurements of pure β-galactosidase. The protein was not measurably taken up by the nucleus within 40 min (Figure 3a).

The basic targeting activity of the karyophilic sequence within the molecular context of our hybrid proteins was studied with protein P1K which contained the minimum signal, i.e. SV40 T-antigen residues 126–132. During the measuring time of 40 min no significant uptake of P1K by the nucleus was observed (Figure 3b). However, on an extended time scale evidence for nuclear uptake was obtained. Thus, 90 min after injection the *Fn/c* ratio was found to be 1.71 ± 0.25 (mean ± SD of five measurements); 140 min after injection the *Fn/c* ratio was 2.44 ± 0.41 (mean ± SD of three measurements). The threonine-variant of P1 was not measurably taken up during the 40-min interval (Figure 3c). Moreland *et al.* (1985) observed that a hybrid protein consisting of the yeast ribosomal protein L3 and β-galactosidase would only be transported from cytoplasm to nucleus if a linker coding for eight amino acid residues was inserted between L3 and the β-galactosidase moiety. Therefore we constructed protein P2K which has a spacer of 11 amino acid residues between the karyophilic sequence and β-galactosidase. However, as shown in Figure 3d, uptake by the nucleus was not noticeably enhanced.

The role of sequences flanking the karyophilic signal was examined with proteins P3 and P4 respectively. In P3 the T-antigen fragment extends towards the C-terminus to include residues 127–147. Again, no indications of transport from cytoplasm to nucleus for the lysine (Figure 3e) or the threonine (Figure 3f) variant were obtained.

In P4 the T-antigen fragment extends towards the N-terminus to contain residues 111–135. In this case (Figure 3g) the lysine variant was extremely rapidly and efficiently taken up by the nucleus. The *Fn/c* ratio reached a maximum of ~15 within 20 min. The half-time of nuclear transport was 8–10 min. The threonine variant of P4 was not noticeably transported within 40 min after injection.

Lateral mobility of the hybrid proteins in cytoplasm and nucleus

Lateral mobility (Table I) was measured by fluorescence microphotolysis (for review see Peters, 1986). The recovery curves were analyzed for two components, a mobile fraction R with an apparent lateral diffusion coefficient D, and an immobile fraction (i.e. $1 - R$). It may be stressed that this type of analysis understands immobility as a relative expression relating to the time scale of the measurement (~1 min in the present case).

In the cytoplasm all hybrid proteins had, within experimental accuracy, about the same D- and R-values. No significant difference between hybrid proteins and pure β-galactosidase was observed. For comparison the mobility of a dextran which has been used previously to probe intracellular aqueous viscosity (Lang *et al.*, 1986) was also measured. The D-value of the dextran (150 kd) was only twice that of the hybrid proteins (480 kd). A difference seemed to exist, however, between the mobile fraction of the hybrid proteins (75%) and that of the dextran (91%). For protein P4K intranuclear mobility was measured

Table I. Lateral mobility of the hybrid proteins[a]

Molecule	Cytoplasm[b]		Nucleus[b]	
	R (%)	*D* ($\mu m^2/s$)	*R* (%)	*D* ($\mu m^2/s$)
P1K	74 ± 10 (8)	0.99 ± 0.39 (20)		
P1T	72 ± 9 (15)	0.92 ± 0.27 (36)		
P2K	74 ± 13 (18)	1.14 ± 0.37 (36)		
P3K	78 ± 7 (9)	1.18 ± 0.19 (25)		
P4K	67 ± 8 (8)	1.09 ± 0.32 (16)	68 ± 10 (33)	0.69 ± 0.21 (51)
P4T	79 ± 7 (18)	1.01 ± 0.22 (37)		
β-Gal[c]	80 ± 9 (12)	0.91 ± 0.26 (22)		
FD150[d]	91 ± 6 (9)	1.95 ± 0.42 (22)		

[a]Mean ± SD, number of measurements is given in parentheses.
[b]*R*, mobile fraction; *D*, apparent lateral diffusion coefficient of mobile fraction.
[c]β-Gal, β-galactosidase.
[d]FD150, fluorescein-labeled 150 kd dextran.

~20 min after cytoplasmic injection, i.e. when nuclear accumulation was virtually completed. The mobile fraction of P4K was the same in cytoplasm and nucleus; the nuclear diffusion coefficient was ~30% smaller than the cytoplasmic one.

The mobility measurements showed that neither the retention of T-variants in the cytoplasm nor the accumulation of the K-variants in the nucleus were based on an association with immobile cytoplasmic or nuclear structures. Theoretical considerations (Horowitz *et al.*, 1970; Elson and Reidler, 1979) suggest that the reversible association of a primarily mobile ligand with immobile binding sites shows up in mobility measurements as a reduction of the apparent lateral diffusion coefficient, as an increase of the immobile fraction, or as a combination of both effects. In the case of P4K, for instance, the mobile fraction was the same in cytoplasm and nucleus. The apparent lateral diffusion coefficient was reduced by 30% in the nucleus which would suffice to explain a 1.5-fold but not a 15-fold enrichment.

Discussion

The present study analyzed the selective transport of large proteins from cytoplasm to nucleus in single living cells by quantitative laser fluorescence microscopy. The basic role of the previously defined (Kalderon *et al.*, 1984a) karyophilic sequence of the SV40 T-antigen, i.e. T-antigen residues 126–132, was confirmed. The sequence appears to be necessary and sufficient to localize large proteins to the nucleus. Our microfluorimetric measurements revealed, however, that the kinetics of nuclear transport are modulated in a rather dramatic manner by T-antigen residues 111–125. The possibility that differences in subcellular distribution and transport kinetics were determined by differential association with immobile structures of cytoplasm or nucleus was ruled out by mobility measurements employing the fluorescence microphotolysis (photobleaching) technique.

Region 111–125 of the SV40 T-antigen has the sequence SSDDEATADSQHSTP. Except for a histidine residue, it does not contain basic residues or sequences which resemble any of the karyophilic sequences defined previously. Thus, it appears improbable that the region contains a second karyophilic signal which could explain the strong effect on nuclear transport kinetics. The region is distinguished, however, by phosphorylation sites. It is therefore appropriate to reconsider a potential participation of phosphorylation in nuclear transport.

Two clusters of phosphorylation sites have been identified in the T-antigen by phosphopeptide mapping (Scheidtmann *et al.*, 1982). The clusters are localized at opposing ends of the peptide chain including residues 106, 111, 112, 123 and 124, at the one end, and residues 639, 676, 677, 679 and 701, on the other end. The functional significance of these sites has remained a complete mystery although some details have been resolved. The phosphorylation state of the T-antigen correlates with its degree of oligomerization (Fanning *et al.*, 1981; Greenspan and Carrol, 1981; Bauman and Hand, 1982; Scheidtmann *et al.*, 1984b), with its affinity for the viral origin of replication (Scheidtmann *et al.*, 1984a; Mohr *et al.*, 1987), and with its ability to support DNA replication *in vitro* (Mohr *et al.*, 1987). With regard to nuclear transport it is particularly relevant that nuclear forms of the T-antigen are more extensively phosphorylated than cytoplasmic ones (Lanford and Butel, 1980; Jarvis *et al.*, 1984; Scheidtmann *et al.*, 1984b) and that phosphorylation of newly synthesized T-antigen seems to proceed in a strict temporal and topographical order (Scheidtmann *et al.*, 1984b). T-antigen is first phosphorylated in the cytoplasm at residues 111, 124 and 701; the residual sites, including residues 112 and 123, are phosphorylated in the nucleus. Speculating, now, on the participation of phosphorylation in nuclear transport several alternative mechanisms are conceivable. (i) Cytoplasmic phosphorylation of residues 111 and 124 may enhance translocation of the T-antigen through the nuclear pore complex, for instance by imposing a favorable conformation or charge distribution on the karyophilic sequence. (ii) Phosphorylation of residues 112 and 123 may be an integral part of the translocation step. (iii) Residues 112 and 123 may be phosphorylated inside the nucleus. This could alter, for instance, the conformation of the karyophilic sequence to render it ineffective for the translocation step and thereby trapping the protein inside the nucleus. Of course, combinations of (i) and (ii) or of (i) and (iii) are also conceivable. Additional hybrid proteins with mutated phosphorylation sites are currently constructed by us to test for these possibilities.

In the past, nuclear protein transport has been studied most extensively in the case of certain amphibian oocyte proteins, in particular nucleoplasmin and N1 (for review, see Dingwall and Laskey, 1986). Is there any evidence for a potential involvement of phosphorylation in these cases? Nuclear transport of nucleoplasmin seems to be induced by a domain of the molecule, which consists of ~20 residues (Dingwall *et al.*, 1988), comprising a tract of four lysine residues

Table II. Structure of the hybrid proteins

Protein	Plasmid	1. Linker	ΔT[a]	2. Linker	ΔGal[b]
P1K	pPR22	MRNSSSV	126–132 (128 = *K*)	GDP	9–1023
P1T	pPR23	MRNSSSV	126–132 (128 = *T*)	GDP	9–1023
P2K	pPR24	MRNSSSV	126–132 (128 = *K*)	GDPLESTCRHA	6–1023
P3K	pPR7	MRNSA	127–147 (128 = *K*)	P	6–1023
P3T	pPR8	MRNSA	127–147 (128 = *T*)	P	6–1023
P4K	pPR16	MRNSSSVRTRGS	111–135 (128 = *K*)	RNSSSPGDP	9–1023
P4T	pPR17	MRNSSSVRTRGS	111–135 (128 = *T*)	RNSSSPGDP	9–1023

[a]ΔT, amino acid residues of the SV-40 T-antigen fragment.
[b]ΔGal, amino acid residues of β-galactosidase.

(Bürglin and De Robertis, 1987). The domain contains also two potential phosphorylation sites, a threonine and a serine, which are spaced from the lysine tract by 6 and 17 residues respectively (in two shorter constructs with nuclear localization the serine is deleted but the spacer contains a serine at a similar position). Nucleoplasmin is a phosphoprotein and occurs in various phosphorylation states (Krohne and Franke, 1980) but the phosphorylation sites have not yet been identified. Nuclear transport of N1 requires a domain of 24 residues (Kleinschmidt and Seitner, 1988). The domain comprises a tract of seven predominantly basic residues which has a close homology to the karyophilic sequence of the SV40 T-antigen. The domain contains also potential phosphorylation sites, two serines, which are spaced from the tract of basic residues by three and 13 residues respectively. A very similar situation is given in the SV40 T-antigen where the established phosphorylation sites are spaced from the karyophilic sequence by two–three and 13–14 residues respectively.

The karyophilic sequence of the SV40 T-antigen is relatively short and simple. Tracts of three–five basic amino acid residues are not a singular feature of large nuclear proteins but do occur in other nuclear and in cytoplasmic proteins (Smith *et al.*, 1985). Together with the outlined observations concerning nucleoplasmin and N1 our results raise the possibility that nuclear protein transport involves more complicated molecular signals, namely domains of some 20 residues in which a tract of predominantly basic residues is spaced from phosphorylation sites by 2–6 and 13–17 residues respectively. The tract of basic residues seems to be necessary and frequently sufficient for nuclear transport. The phosphorylation sites might add a great deal of selectivity and efficiency to the system.

Independently of any speculations our study has shown that a characterization of karyophilic sequences should include the complete transport kinetics and not just equilibrium distributions. Our observation that nuclear transport kinetics can be largely enhanced by adding to the karyophilic signal certain flanking sequences could be of decisive importance for practical applications, e.g. drug targeting.

Materials and methods

Media and reagents

LB-broth and TY-medium were used as described by Maniatis *et al.* (1982). Ampicillin (100 μg/ml) or tetracyclin (25 μg/ml) were added to media and plates. X-gal was dissolved in formamide and added to plates at a final concentration of 40 μg/ml. Isopropyl-β-D-thiogalactoside (IPTG) was used at concentrations of 0.3–1.0 mM in media and plates to induce expression of β-galactosidase and fusion proteins. The *E.coli* strains JM105 (Yanisch-Perron *et al.*, 1985) or MC1060 (Shapira *et al.*, 1983) were used. T4–DNA-ligase, DNA modifying and restriction enzymes were used as specified by the commercial suppliers. DNA transformation (Dagert and Ehrlich, 1979), DNA agarose and polyacrylamide gels (Maniatis *et al.*, 1982) and DNA sequence analysis (Sanger *et al.*, 1977) were performed according to published protocols.

Construction of plasmids

Only a short description of the plasmid constructions will be given here as details have been given elsewhere (Rihs, 1989). Table II contains information on nomenclature and predicted primary structure of the hybrid proteins.

pPR7 and pPR8. The *lacZ* gene of pMC1871 was set as a *Bam*HI fragment into the *Bam*HI linearized expression vector pHK255 (Kröger and Hobom, 1987) to yield pPR2. The large *Hin*dIII–*Bam*HI fragment of L7RH or d10L7RH (Kalderon *et al.*, 1984b) was ligated with the small *Hin*dIII–*Bam*HI fragment of d10L27βGAL (Kalderon *et al.*, 1984a,b) to yield pPR3 or 4 respectively. The small *Eco*RI fragment of pPR3 or pPR4 respectively, was ligated with the large *Eco*RI fragment of pPR2 yielding pPR7 or pPR8 respectively.

pPR16 and pPR17. L27βGAL or d10L27βGAL were digested with *Pfl*MI, 3′-protruding ends were removed by T4 DNA polymerase and *Eco*RI linker dCGGAATTCCG was added. The *Eco*RI fragment coding residues 87–135 of the SV40 T-antigen was isolated and cloned into *Eco*RI partially digested pPR2 yielding pPR11 or pPR12 respectively. pPR11 or pPR12 was digested with *Mae*I (SV40 nucleotide 4483), filled up by dNTP, and set into the *Sna*BI site of the expression vector pHK402 (Kröger and Hobom, 1987). The reading frame of the resulting plasmids pPR13 or pPR14 was adjusted by *Spe*I and *Xba*I digestion and by cloning of the small fragment into *Xba*I-digested pHK402. This yielded pPR16 or pPR17 respectively.

pPR22, pPR23 and pPR24. A mixed oligonucleotide 5′dAAAA(A/C) GAAGAGAAAGGTA3′ was cloned into *Sma*I-digested M13mp18-RF. Clones coding for lysine or threonine in SV40 T-antigen position 128 were identified by sequencing. The RF-forms of the clones were digested with *Eco*RI–*Hin*dIII. The small fragment was cloned into pHK255 yielding pPR20 or pPR21 respectively. The *lacZ* gene of pMC1871 was cloned as *Bam*HI fragment into *Bam*HI-linearized pPR20 or pPR21 yielding pPR22 or pPR23 respectively. The *lacZ* gene of pPR7 was cloned as *Hin*dIII fragment into the unique *Hin*dIII site of pPR20 yielding pPR24.

Expression and purification of the hybrid proteins

Bacterial cultures were grown up in 500 ml LB-broth in the presence of 0.3–1.0 mM IPTG and 100 μg/ml ampicillin until the OD at 600 nm was 0.8. Bacteria were harvested by centrifugation, suspended in 5 ml of buffer A containing 20 mM Tris, pH 7.4, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and disrupted by sonication at 0°C. After centrifugation (38 000 *g*, 30 min, 4°C) the supernatant was collected. NaCl was added to a final concentration of 1.6 M. The supernatant was then loaded on a column of *p*-amino-phenyl-β-D-thiogalactoside–Sepharose, synthesized according to Ullmann (1984). The column was extensively washed with buffer A plus NaCl (final concentration 1.6 M) until non-specifically bound material had been completely eluted as indicated by the OD at 280 nm. The hybrid

proteins were eluted in small fractions with a 100 mM sodium borate buffer, pH 10.05 containing 10 mM 2-mercaptoethanol. Eluted fractions were immediately neutralized by titration with 2 M Tris to pH 7.4. Aliquots of the eluted fractions were analysed by SDS–PAGE and assayed for protein concentration and β-galactosidase activity. For further use the fractions were stored at −20°C.

Fluorescent labeling of hybrid proteins

Proteins (0.4–4 mg/ml) were dialysed against 1.5 l of a solution containing 20 mM Tris, pH 7.4 and 10 mM $MgCl_2$ for 2 h at 8°C. The solution was adjusted to pH 7.8 by titration with a 250 mM solution of *N,N*-bis(2-hydroxyethyl)-glycine (Bicine), pH 8.0. A solution containing 4 mg/ml of the sulfhydril-reactive fluorophore, IAF (Molecular Probes, Junction City, OR, USA), in 250 mM Bicine, pH 8.0 was freshly prepared. Protein and fluorophore solutions were mixed at a volume ratio of 9:1 and incubated for 60 min at 22°C in the dark. The reaction was terminated by addition of 2-mercaptoethanol. Unreacted IAF was removed from the protein solution by extensive pressure filtration (Amicon cell with PM10 filter). The ratio of protein-bound to unreacted free IAF was determined by running samples on 10% SDS–polyacrylamide gels and scanning the gels for fluorescence. Unreacted IAF always amounted to <10% of protein-bound IAF. The labeling ratio (i.e. moles IAF/mole of protein) was measured spectrophotometrically according to Goldman (1968).

Mammalian cells, microinjection and laser microscopy

HTC cells, a derivative of Morris hepatoma 7288C, were obtained from Flow Laboratories (Bonn, FRG) and fused with PEG to yield polykaryons as described (Lang *et al.*, 1986). For microinjection the hybrid proteins were always dissolved at a concentration of ~2.7 mg/ml and introduced into the cytoplasm of these cells by pressure microinjection using the microinjector of Eppendorf (Hamburg, FRG) and micromanipulators by Leitz (Wetzlar, FRG) or Zeiss (Oberkochen, FRG).

The methods used to measure lateral molecular mobility and transport across membranes by fluorescence microphotolysis have been described (Peters, 1986). In the present case a circular field of 2 μm radius was employed in mobility measurements. The same field size was also used to measure fluoresence in small areas of nucleus and cytoplasm from which measurements the nucleocytoplasmic fluorescence ratio *Fn/c* was derived. Further experimental details are found in Lang *et al.* (1986) and Schulz and Peters (1987). All measurements were performed at 37°C.

Histochemical assay of β-galactosidase activity

Hybrid proteins were injected into HTC cells. Fifteen hours after injection cells were washed three times with phosphate-buffered saline (PBS) and fixed with 2% (v/v) formaldehyde, 0.2% (v/v) glutaraldehyde in PBS and incubated with X-gal as described by Sanes *et al.* (1986). After relocation of the injected cells in the microscope micrographs were taken under through-light illumination.

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