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The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen

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We have previously demonstrated [Rihs,H.-P. and Peters,R. (1989) *EMBO J.*, 8, 1479-1484] that the nuclear transport of recombinant proteins in which short fragments of the SV40 T-antigen are fused to the amino terminus of *Escherichia coli* β-galactosidase is dependent on both the nuclear localization sequence (NLS, T-antigen residues 126-132) and a phosphorylation-site-containing sequence (T-antigen residues 111-125). While the NLS determines the specificity, the rate of transport is controlled by the phosphorylation-site-containing sequence. The present study furthers this observation and examines the role of the various phosphorylation sites. Purified, fluorescently labeled recombinant proteins were injected into the cytoplasm of Vero or hepatoma (HTC) cells and the kinetics of nuclear transport measured by laser microfluorimetry. By replacing serine and threonine residues known to be phosphorylated *in vivo*, we identified the casein kinase II (CK-II) site S^{111}/S^{112} to be the determining factor in the enhancement of the transport. Either of the residues 111 or 112 was sufficient to elicit the maximum transport enhancement. The other phosphorylation sites (S^{120}, S^{123}, T^{124}) had no influence on the transport rate. Examination of the literature suggested that many proteins harboring a nuclear localization sequence also contain putative CK-II sites at a distance of ~10-30 amino acid residues from the NLS. CK-II has been previously implicated in the transmission of growth signals to the nucleus. Our results suggest that CK-II may exert this role by controlling the rate of nuclear protein transport.

Key words: nuclear cytoplasmic transport/protein phosphorylation/regulation/SV40 T-antigen

Introduction

Recently, it has been found that changes in the proliferation and differentiation status of eukaryotic cells may be accompanied by the selective relocalization of proteins between cytoplasm and nucleus. This holds, for instance, for the *fos*- and *rel*-encoded oncoproteins (Roux *et al.*, 1990; Capobianco *et al.*, 1990), certain transcription factors (Lenardo and Baltimore, 1989; Shirakawa and Mizel, 1989; Ghosh *et al.*, 1990), the *Drosophila* morphogen *dorsal* (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989), and the steroid hormone receptor (Kost *et al.*, 1989). Such observations lend new exciting support to an old but hitherto largely unexplored hypothesis according to which the nuclear cytoplasmic transport of proteins which are able to interfere with replication and/or transcription, constitutes a regulatory level in cellular growth and differentiation.

Some of the basic facts underlying nuclear cytoplasmic protein transport have been elucidated in recent years (for review, see Dingwall and Laskey, 1986; Peters, 1986; Newport and Forbes, 1987; Gerace and Burke, 1988; Roberts, 1989). Most noticeably, it has been shown that the transport of proteins with a mass larger than ~50 kd requires specific signal sequences (Kalderon *et al.*, 1984; Lanford and Butel, 1984; Hall *et al.*, 1984; Silver *et al.*, 1984). The best characterized example of a nuclear localization sequence (NLS) is that of the Simian virus 40 large tumor antigen (SV40 T-antigen) which has the structure $PKKKRKV^{132}$ (Kalderon *et al.*, 1984). NLSs have since been identified in more than 30 proteins. However, a strict consensus sequence has not emerged and only some general rules seem to apply: the NLS is usually short, contains a high proportion of basic residues, does not need to be located at specific sites within the nuclear-targeted protein, and is an integral part of the protein not removed during transport.

Although the specificity of nuclear cytoplasmic transport is apparently determined by NLSs the kinetics of transport may be regulated by quite different parameters. In a preceding study (Rihs and Peters, 1989) we observed that the NLS of the SV40 T-antigen is necessary and sufficient for inducing the nuclear accumulation of large recombinant proteins. However, the rate of nuclear transport was dramatically enhanced if, in addition to the NLS, the T-antigen residues 111-125 were present. Since this flanking region harbors a cluster of phosphorylation sites (S^{111}, S^{112}, S^{120}, S^{123} and T^{124}; Scheidtmann *et al.*, 1982) we suggested that nuclear cytoplasmic transport kinetics are regulated by phosphorylation of one or several of these sites.

The SV40 T-antigen is a complete oncogene, i.e. it is able, without support by other oncogene products, to transform fully primary cells and induce tumor formation (for review, see Butel, 1986; DePamphilis and Bradley, 1986). The T-antigen contains two clusters of phosphorylation sites which are located at the amino-terminal and the carboxy-terminal parts of the peptide chain, respectively. Much attention has been paid to these sites and several investigations have been directed to elucidate the role of T-antigen phosphorylation, for instance, in viral replication, the synthesis of early and late mRNAs, and the interaction of the T-antigen with tumor suppressor proteins such as p53 or Rb, the product of the retinoblastoma susceptibility gene. According to present knowledge (reviewed by Prives, 1990) the phosphorylation of T^{124} by cdc-2 kinase is sufficient to activate the replication function of the T-antigen *in vitro*, whereas phosphorylation of one or two serine residues appears to inhibit T-antigen function.

In the present study the role of specific phosphorylation

sites in the regulation of nuclear cytoplasmic protein transport was investigated. We constructed a number of recombinant proteins in which, by deletion or oligonucleotide-directed mutagenesis, the phosphorylation sites were selectively replaced by non-phosphorylatable residues. Fluorescence microscopic single-cell measurements of the kinetics of nuclear accumulation of these proteins identified the phosphorylation site which is important for enhancement of nuclear cytoplasmic transport. This turned out to be a casein kinase II (CK-II) site located about 15 amino acid residues amino-terminal of the NLS. Examination of the literature suggested that many proteins harboring a nuclear localization sequence also contain potential CK-II sites at a distance of ~10–30 amino acid residues from the NLS. CK-II has been implicated to play a role in the transmission of growth signals to the nucleus. Thus, our results shed new light on potential mechanisms underlying the implicated role of CK-II and the regulation of cellular proliferation.

Results

Recombinant proteins for studying the role of phosphorylation in nuclear protein transport

We have previously generated recombinant proteins which contain short sequences of the SV40 T-antigen fused to the amino-terminus of *E.coli* β-galactosidase (Rihs and Peters, 1989). The primary structure of proteins P1, P3 and P4 is indicated in Table I. All of these proteins contained the SV40 T-antigen NLS (residues 128–132) either alone (i.e. protein P1) or in conjunction with carboxy-terminal (i.e. P3) or amino-terminal flanking sequences (i.e. P4). For most proteins, variants were generated with either a lysine (K-variants) or a threonine (T-variants) residue in position 128 because substitution of lysine^{128} by threonine completely abolishes the targeting activity of the NLS (Kalderon *et al.*, 1984) and thus serves as a control for the specificity of the NLS.

We generated a new set of proteins, P6–P10 (Table I), all of which contained the wild-type NLS and amino-terminal flanking sequences. The phosphorylation sites in the flanking sequences, however, were exchanged by non-phosphorylatable residues. Thus, in the protein P6K, S^{111} was replaced by G; in P8K both S^{123} and T^{124} were replaced by A; in P9K S^{112} was replaced by G, and in P10K S^{120}, S^{123} and T^{124} were replaced by A. P7K contained only T-antigen residues 120–135 and hence lacked S^{111} and S^{112}. The recombinant proteins were expressed in *E.coli* and isolated by affinity chromatography on a thiogalactoside column. This yielded large tetrameric proteins of ~480 kd displaying β-galactosidase activity.

Cells for the study of nuclear cytoplasmic protein transport

In preceding studies we characterized the intracellular transport of macromolecules in polykaryons generated by treating rat hepatoma tissue culture (HTC) cells with polyethylene glycol (Lang *et al.*, 1986; Schulz and Peters, 1987; Rihs and Peters, 1989). Polykaryons are easily injected, have a large nuclear cytoplasmic volume ratio, and are arrested in interphase. This can be an advantage because the disassembly and reconstruction of the nuclear envelope at mitosis interfere with long-term studies of nuclear cytoplasmic transport. Meanwhile, however, we became aware

Table I. Primary structure of SV40 T-antigen β-galactosidase fusion proteins[a]

Protein	Phosphorylation Sites (111 112 120 123 124)
P1K	MRNSSSV**PKKKRKV**GDP
P3K	MRNSA**KKKRKV**EDPKDFPSELLSFLSP
P4K	MRNSSSVRTRGSSSDDEATADSQHSTP**PKKKRKV**EDPRNSSSPGDP
P4T	MRNSSSVRTRGSSSDDEATADSQHSTP**PKTKRKV**EDPRNSSSPGDP
P6K	MRNSSSPGGSDDEATADSQHSTP**PKKKRKV**EDPRNSSSPGDP
P7K	MRNSSSVRTHSQHSTP**PKKKRKV**EDPRNSSSPGDP
P8K	MRNSSSVRTRGSSSDDEATADSQHAAP**PKKKRKV**EDPRNSSSPGDPLESTCSP-
P9K	MRNSSSVRTRGSGDDEATADSQHSTP**PKKKRKV**EDPRNSSSPGDP
P10K	MRNSSSVRTRGSSSDDEATADAQHAAP**PKKKRKV**EDPRNSSSPGDPLESTCSP-

[a]One-letter amino acid code is used. SV40 T-antigen residues are underlined. The nuclear localization sequence is set in bold. The bar indicates *E.coli* β-galactosidase residues 6/9–1023.

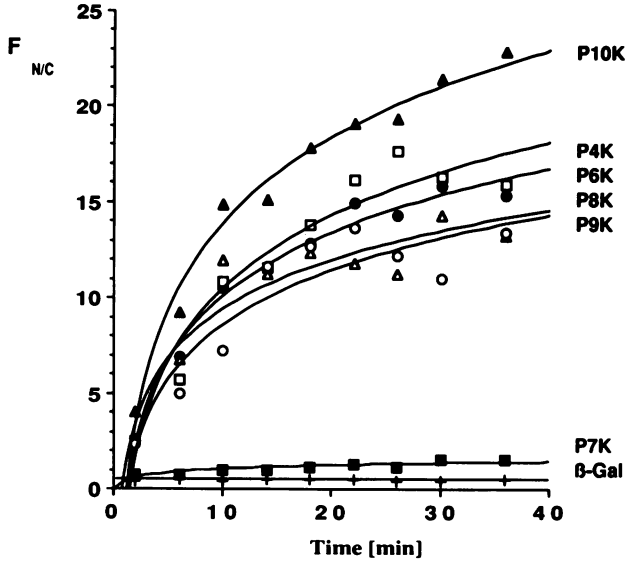

Fig. 1. Effect of phosphorylation site mutation on the nuclear cytoplasmic transport of recombinant proteins in HTC polykaryons. Fluorescently labeled recombinant proteins were injected into the cytoplasm of HTC polykaryons. The nuclear cytoplasmic fluorescence ratio $F_{n/c}$ was determined by microfluorimetry and plotted versus time after injection. Each data point is the average of 5–10 measurements. The curves were obtained by fitting an exponential function to the data. Injected proteins (see Table I) were: P4K (□), P6K (●), P7K (■), P8K (△), P9K (○), P10K (▲), and β-galactosidase (+).

of parameters which regulate nuclear cytoplasmic transport. Because these mechanisms might well be coupled to the cell cycle we have now extended our studies to normally dividing cells, choosing the Vero cell line, which is derived from African green monkey kidney and widely employed in SV40 studies.

The effect of phosphorylation site mutation on nuclear cytoplasmic transport in HTC polykaryons

Isolated recombinant proteins were labeled with the SH-reactive fluorescent probe 5-iodoacetamidofluorescein (IAF) and injected with a micropipette into the cytoplasm of single

HTC polykaryons. The kinetics of nuclear cytoplasmic transport during the first 40 min after injection were measured by laser microfluorimetry as described previously (Peters, 1986). The long-term (~12 h) accumulation of cytoplasmically injected proteins in the nucleus was assessed (Rihs and Peters, 1989) by the histochemical 'X-gal' assay which makes use of the β-galactosidase activity of the recombinant proteins.

Short-term kinetics of nuclear cytoplasmic protein transport are displayed in Figure 1. The nuclear cytoplasmic fluorescence ratio $F_{n/c}$ is plotted as a function of the time after injection. The previous observation (Rihs and Peters, 1989) was confirmed that of proteins P1K–P4K only P4K was rapidly transported into the nucleus, reaching an $F_{n/c}$ value of ~15 within 40 min. P1K and P3K were only very slowly taken up by the nucleus ($F_{n/c} \approx 1-2$ after 40 min) (not shown) while all of the T-variants as well as β-galactosidase remained in the cytoplasm ($F_{n/c} < 1$). Of proteins P6K–P10K, all except P7K were rapidly accumulated in the nucleus, reaching $F_{n/c}$ values of 12–22 within 40 min. In stark contrast P7K reached a maximum $F_{n/c}$ of only 1–2.

In spite of their largely different short-term kinetics all proteins containing the wild-type NLS were eventually accumulated in the cell nucleus. For instance, the nuclear accumulation of P7K was completed only after ~12 h as assessed by the X-gal assay (Figure 2A–D). P10K, on the other hand, was perfectly accumulated in the nucleus within 10 min as shown by both fluorescence micrographs of living cells (Figure 2E) and the histochemical assay (Figure 2F).

In Vero cells the kinetics of nuclear protein transport depend on phosphorylation sites

The short-term kinetics of nuclear protein accumulation in Vero cells are shown in Figure 3. Whereas P1K was not noticeably transported during ~40 min, P4K reached a $F_{n/c}$ of ~15 in the same time. P4T remained strictly cytoplasmic ($F_{n/c} < 1$). P3K, with a maximum $F_{n/c}$ of 4, behaved in a similar fashion to P1K. As in HTC polykaryons, P6K and P10K were rapidly transported ($F_{n/c}$ 12–15 after 40 min) whereas the nuclear accumulation of P7K was rather slow ($F_{n/c} \approx 2$ after 40 min). Nevertheless, the X-gal assay revealed that P7K was completely taken up by the nucleus on a time scale of several hours (Figure 4C–F).

Identification of phosphorylation sites responsible for the enhancement of nuclear cytoplasmic transport

In Table II the recombinant proteins are arranged with respect to phosphorylation sites. This is correlated with both their short-term (40 min) and long-term (~12 h) accumulation in the nucleus as assayed by microfluorometry or the X-gal assay, respectively.

A comparison of K- and T-variants of the recombinant proteins confirms previous conclusions (Kalderon *et al.*, 1984; Rihs and Peters, 1989) that the SV40 T-antigen NLS is necessary and sufficient for nuclear protein accumulation. This holds for both HTC polykaryons and Vero cells. Thus, the specificity of nuclear cytoplasmic protein transport is determined by the NLS.

However, the kinetics of nuclear accumulation clearly depended on the phosphorylation sites contained in the sequence flanking the NLS on its amino-terminal border. For recombinant proteins which contained either no phosphorylation sites at all (P3K), or the sites T^{124} and S^{123} (P1K), or T^{124}, S^{123} and S^{120} (P7K) short-term accumulation was slow. 30 min after injection, the $F_{n/c}$ values were either well below 1 (P3K, P1K) or maximally 1–2 (P7K). This behavior changed abruptly when either the phosphorylation site S^{112} (P6K) or S^{111} (P9K) was present. For these proteins the $F_{n/c}$ was >12 forty minutes after injection. The

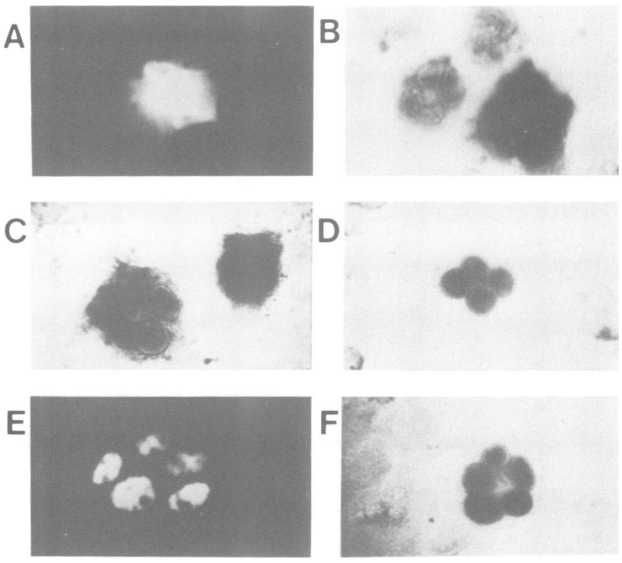

Fig. 2. Long-term kinetics of nuclear protein transport in HTC polykaryons. Fluorescently labeled proteins were injected into the cytoplasm of HTC polykaryons. The subcellular distribution of recombinant proteins was assessed either by fluorescence or, after fixation and processing, by the X-gal assay. (**A–D**) P7K at 10 min (A, fluorescence and B, X-gal), 90 min (C, X-gal), and ~12 h (D, X-gal) after injection. (**E,F**) P10K at 10 min after injection (E, fluorescence and F, X-gal).

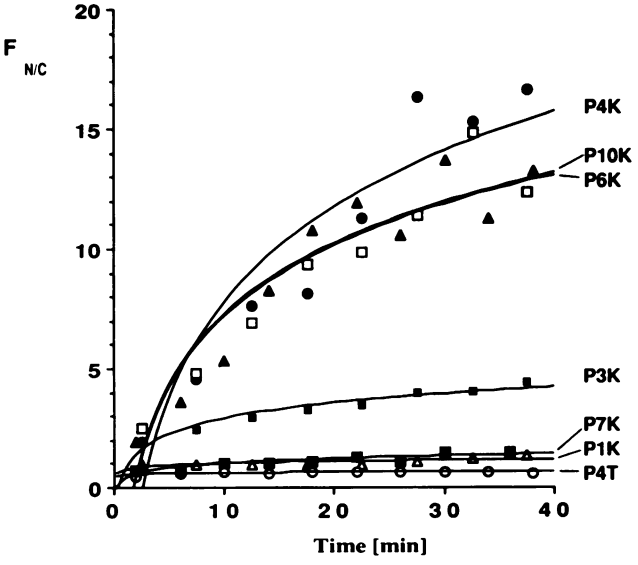

Fig. 3. Nuclear cytoplasmic transport in Vero cells. Fluorescently labeled recombinant proteins were injected into the cytoplasm of Vero cells. The nuclear cytoplasmic fluorescence ratio $F_{n/c}$ was determined by microfluorimetry and plotted versus time after injection. Each data point is the average of 5–10 measurements. The curves were obtained by fitting an exponential function to the data. Injected proteins (see Table I) were: P1K (△), P3K (■), P4K (□), P4T (○), P6K (●), P7K (■), P10K (▲).

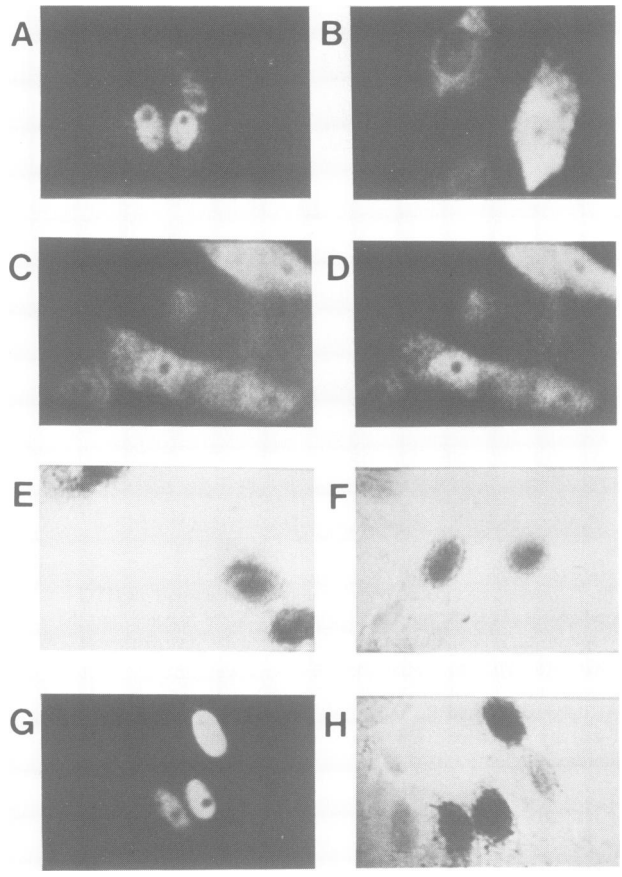

Fig. 4. Long-term kinetics of nuclear protein transport in Vero cells. Fluorescently labeled proteins were injected into the cytoplasm of Vero cells. The subcellular distribution of recombinant proteins was assessed either by fluorescence or, after fixation and processing, by the X-gal assay. (**A**) P4K, 10 min after injection, fluorescence. (**B**) P4T, 40 min after injection, fluorescence. (**C–F**) P7K at 10 min (C, fluorescence), 40 min (D, fluorescence), 40 min (E, X-gal), and ~12 h (F, X-gal) after injection. (**G, H**) P10K at 10 min after injection (G, fluorescence and H, X-gal).

presence of both sites, S^{112} and S^{111}, as in P4K, did not significantly accelerate nuclear transport above the level achieved by one site alone. On the other hand, if S^{112} and S^{111} were present, T^{124} and S^{123} (P8K) or T^{124}, S^{123} and S^{120} (P10K) could be substituted by non-phosphorylatable residues without reduction of the nuclear transport rate. Thus, the S^{111}/S^{112}-site was the determining factor for the transport kinetics.

It has been pointed out previously (Krebs *et al.*, 1988) that the T-antigen sequence Ser-Ser-Asp-Asp-Glu^{115} optimally fulfils the specific sequence requirements for a CK-II phosphorylation site (for review, see Hathaway and Traugh, 1982; Pinna *et al.*, 1985; Edelman *et al.*, 1987; Krebs *et al.*, 1988). In fact, purified CK-II phosphorylates SV40 T-antigen *in vitro* and phosphopeptide mapping showed that phosphorylation is at S^{106} and S^{112} (Grässer *et al.*, 1988).

The CK-II-site/spacer/NLS motif occurs in several proteins

Is the CK-II-site/spacer/NLS motif also present in proteins other than the SV40 T-antigen? We inspected several NLS-containing proteins (Table III). The comparison included most proteins whose NLS was tested by the ability of an analogous synthetic peptide to target a large inert protein to the nucleus (Chelsky *et al.*, 1989). In addition, a number of other, particularly well studied proteins were included. In each case a site conforming to the consensus CK-II recognition sequence S/T-X-X-D/E (Krebs *et al.*, 1988) was found in the close neighborhood of the NLS. In several cases (6 out of 16) the motif was reversed (NLS/spacer/CK-II-site). The average distance between CK-II site and NLS was 23 ± 12 amino acid residues (mean ± SD, n = 16). In the case of the best characterized proteins (SV40 T-antigen, polyoma virus T-antigen, nucleoplasmin, N1/N2-protein) the length of the spacer was 15 ± 10 amino acid residues. Thus, CK-II sites may be present in most or all proteins carrying a NLS with a potential close topographical relation between the CK-II site and NLS.

Table II. Relationship between phosphorylation sites and nuclear accumulation of SV40 β-galactosidase fusion proteins in HTC polykaryons and Vero cells

Protein	Residue[a]						Nuclear cytoplasmic concentration ratio[b]	
	111	112	120	123	124	128	30 min	~12 h
P3K	–	–	–	–	(N)	K	<1	Nuc
P1K	–	–	–	(S)	(S)	K	<1	Nuc
P7K	(R)	(N)	S	S	T	K	1–2	Nuc
P6K	G	S	S	S	T	K	>12	Nuc
P9K	S	G	S	S	T	K	>12	Nuc
P4K	S	S	S	S	T	K	>12	Nuc
P4T	S	S	S	S	T	T	<1	Cyt
P8K	S	S	S	A	A	K	>12	Nuc
P10K	S	S	A	A	A	K	<12	Nuc

[a]The underlined residues are SV40 T-antigen wild type residues. Residues in brackets are part of the linkers.
[b]The nuclear cytoplasmic concentration ratio was estimated either from the nuclear cytoplasmic fluorescence ratio $F_{n/c}$ (30 min after injection) or from the X-gal assay (~12 h after injection) where Nuc and Cyt mean virtually complete nuclear or cytoplasmic localization, respectively.

Discussion

The present study identified residues S^{111}/S^{112} of the SV40 T-antigen as the factor determining the kinetics of nuclear cytoplasmic protein transport. Surprisingly, the presence of either one of these residues is sufficient to elicit the maximum enhancement of nuclear transport. Recent *in vitro* studies (Grässer *et al.*, 1988) suggested that only S^{112}, but not S^{111}, is phosphorylated by purified CK-II. However, our result, if applicable to authentic T-antigen, would imply that biological responses which might be associated with the enhancement of nuclear transport by S^{111}/S^{112} phosphorylation can only be disturbed and thus detected if both residues are replaced. Previous studies bearing on the function of T-antigen phosphorylation have frequently substituted only one of the two residues (e.g. Kalderon and Smith, 1984; Chen and Paucha, 1990).

We have recently developed (Ackermann,M., Jans,D.A. and Peters,R., unpublished results) a model system for the study of nuclear cytoplasmic transport which is based on the

Table III. Relationship between nuclear localization sequences and potential casein kinase II sites

Protein	Predicted NLS[a]	Putative CK-II site[b]	Distance[c]
SV40 T	PKKKRKV^{132} (1)	SSDDE^{115}	−13
Polyoma T	VSRKRPRP^{196} (2)	SSPTD^{159}	−32
	PPKKARED^{286} (2)	ESENE^{264}	−17
Nucleoplasmin	RPAATKKAGQAKKKKLD^{172} (3)	EDESSEED^{180}	+4
N1/N2	VRKKRKT^{537} (4)	SAVE^{511}	−22
	AKKSKQEP^{554} (4)	TEEE^{540}	−10
Lamin L_I	VRTTKGKRKRIDV^{420} (5)	SIEE^{444}	+21
Lamin A/C	SVTKKRKLE^{422} (6)	SNED^{461}	+35
c-Myc	PAAKRVKLD^{328} (7)	SSDTEE^{352}	+19
	RQRRNELKRSF^{374} (7)	SSDTEE^{352}	+15
Ad7 E1a	STRKLPRQ^{261} (8)	SDDE^{201}	−56
SV40 VP1	APTKRKGS^{8} (9)	SFTEVE^{48}	+34
Human p53	PQPKKKPL^{323} (8)	TEEE^{287}	−31
NF-$\varkappa$B p50	QRKRQK^{372} (10)	SDLETSE^{350}	−22
Mouse c-rel	KSKKQK^{295} (11)	SDQEVSE^{273}	−22
Human c-rel	KAKKQK^{295} (11)	SDQEVSE^{273}	−22

[a]Reference for NLS: (1) Kalderon *et al.* (1984); (2) Richardson *et al.* (1986); (3) Dingwall *et al.* (1988); (4) Kleinschmidt and Seiter (1988); (5) Krohne *et al.*, 1987; (6) Loewinger and McKeon (1988); (7) Dang and Lee (1988); (8) Chelsky *et al.*, 1989; (9) Wychowski *et al.*, 1986; (10) Ghosh *et al.*, 1990; (11) Gilmore and Temin, 1988.

[b]Sequences conforming to the consensus CK-II recognition site S/T-X-X-D/E (Krebs *et al.*, 1988) were considered, taking into account the fact that additional acidic residues on either side enhance the specificity. Unusual CK-II sites such as VGPDSD^{390} in human p53 (Meek *et al.*, 1990) were not considered.

[c]The distance is given in amino acid residues spacing the phosphorylatable S or T residues of the putative CK-II from the boundary of the predicted NLS.

perforation of cultured cells according to Simons and Virta (1987). The nuclear accumulation of NLS-containing recombinant proteins in the model system resembled that *in vivo* if the perforated cells were incubated with a cytosolic extract of *Xenopus* oocytes. Residues S^{111} and/or S^{112} appeared to be responsible for the enhancement of the transport rate. Only recombinant proteins carrying S^{111} and/or S^{112} were phosphorylated to a marked extent when incubated with a cytosolic extract of *Xenopus* oocytes whilst proteins not containing S^{111} and/or S^{112} (P3, P7, β-galactosidase) were not. These data suggest that it is phosphorylation of S^{111} and/or S^{112} which may directly regulate transport kinetics.

Several mechanisms by which phosphorylation at S^{111}/S^{112} could regulate nuclear transport are conceivable. One major possibility would be that phosphorylation at S^{111}/S^{112} directly affects the NLS. A conformational change of the NLS, induced by phosphorylation at the nearby site, might modulate the affinity of the NLS for its presumptive receptor whose identity and subcellular localization is still controversial (Yoneda *et al.*, 1988; Adam *et al.*, 1989; Benditt *et al.*, 1989; Li and Thomas, 1989; Lee and Mélèse, 1989; Silver *et al.*, 1989; Yamasaki *et al.*, 1989). As a second possibility one might consider that the CK-II site is a primarily independent functional entity. This would be consistent with the notion that the T-antigen is a mosaic of functional domains containing distinct regions for Rb-, p53-, ATP-, and origin-binding (for review, see Prives, 1990). The comparison presented in Table III, however, reveals a potential close topographical relationship between NLSs and CK-II sites which favors the assumption of direct interactions.

The physiological role of CK-II, which does not appear to be mediated by a second messenger, is still largely unresolved. Sommercorn *et al.* (1987) observed that the cellular level of CK-II activity rapidly increased when cells were stimulated by insulin or growth factors. Significantly, most of the proteins encoded by nuclear oncogenes such as *myc*, *myb*, *fos*, or the adenovirus E1a gene harbor potential CK-II sites (Krebs *et al.*, 1988). It is also known (Dyson *et al.*, 1989) that the region of the SV40 T-antigen (residues 101−118) comprising the CK-II site is highly conserved among papilloma viruses. Homologous regions are found in the E1a protein of adenovirus and the E7 protein of human papilloma virus. Barbosa *et al.* (1990) observed recently that the relative oncogenic potential of human papilloma virus subtypes directly correlates with the presence of the phosphorylation site which is homologous to S^{111}/S^{112} of the SV40 T-antigen. On these grounds CK-II was implicated (Krebs *et al.*, 1988; Lüscher *et al.*, 1989) to play a role in the transduction of growth signals to the nucleus. Our results open up the possibility that CK-II may exert this function by regulating the rate of nuclear protein transport. The SV40 T-antigen fragment 111−135 which is present in P4 and some of the other recombinant proteins contains two groups of phosphorylation sites, S^{111}/S^{112} and S^{123}/T^{124}. T^{124} has been shown (McVey *et al.*, 1989) to be specifically phosphorylated *in vitro* by purified cdc-2 kinase. We have recently observed (unpublished results) that our recombinant proteins are specifically phosphorylated by cdc2 kinase from HeLa cells. This opens the possibility for cell cycle dependent interactions between the CK-II sites and the cdc2 sites consistent with CK-II's known role in synergistic phosphorylation mechanisms (Picton *et al.*, 1982; DePaoli-Roach *et al.*, 1983; Woodgett and Cohen, 1984; Fiol *et al.*, 1987).

In conclusion we suggest that direct correlations exist between phosphorylation at CK-II sites, the rate of nuclear protein transport, and the modulation of gene expression. Within this general framework many different mechanisms are still conceivable. With regard to the general role of phosphorylation in cellular regulation it appears very likely

that a complex, yet uncharacterized, network of interacting kinases and phosphorylases is also involved in the regulation of nuclear cytoplasmic transport. By continuing with our approach which combines genetic and biochemical methods with quantitative fluorescence microscopy we should be able to unravel some important functional connections.

Materials and methods

Media and reagents

LB broth and TY medium were prepared according to Maniatis *et al.* (1982). Ampicillin (100 μg/ml) was 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 recombinant proteins. The *E.coli* strain MC1060 (Shapira *et al.*, 1983) was routinely used, together with TG-1 as M13 host. DNA transformation, DNA agarose and polyacrylamide gels and DNA sequencing were performed according to Dagert and Ehrlich (1979), Maniatis *et al.* (1982) and Sanger *et al.* (1977), respectively.

DNA constructs

The construction of plasmids encoding proteins P1–P4 has been described previously (Rihs and Peters, 1989).

Plasmid pPR25, encoding protein P6, was derived from pPR16 following *Mae*I restriction, filling in using DNA polymerase (Klenow), addition of *Sma*I (10mer) linkers, and ligation into *Sma*I restricted plasmid pPR2 vector (Rihs and Peters, 1989). Plasmid pPR27, encoding protein P9, was derived from plasmid pPR16 (Rihs and Peters, 1989) following *Mae*I restriction, exonuclease removal of sticky ends using mung bean nuclease and T4 DNA polymerase, addition of *Bgl*II (8mer) linkers, and ligation into *Bam*HI restricted plasmid pHK402 vector (Kröger and Hobom, 1987). Plasmid pPF10, encoding protein P7, was constructed by initially isolating the 106 bp *Hin*fI fragment of pPR16 containing the SV40 T-antigen sequence and filling in using Klenow, prior to *Bam*HI restriction and isolation of the 71 bp *Hin*fI-filled–BamHI fragment containing SV40 T-antigen residues 120–135. This fragment was ligated with *Sma*I–*Bam*HI restricted plasmid pHK402 to yield plasmid pPF9 (~4.3 kb) and the lacZ-containing *Bam*HI fragment from pPR16 was then inserted into the *Bam*HI site carboxy-terminally adjacent to the SV40 sequences. Plasmids pPF17, encoding protein P8, and pPR28, encoding protein P10, were derived following subcloning of the 108 bp *Eco*RI fragment of pPR16, containing the SV40 sequences, into *Eco*RI-restricted M13 mp19-RF (Yanisch-Perron *et al.*, 1985) to yield the M13 derivative mp19PF14. Oligonucleotide site-directed mutagenesis was performed according to Eckstein and coworkers (Taylor *et al.*, 1985a,b). After mutation, the 108 bp *Eco*RI fragments were subcloned into pPR2 to yield constructs comparable to pPR16. In the case of pPF17 this involved inserting the mutated 108 bp fragment into *Eco*RI restricted plasmid pHK255 (Kröger and Hobom, 1987) to yield plasmid pPF16, followed by insertion of the lacZ-containing *Hin*dIII fragment from the plasmid pPR7 (Rihs and Peters, 1989) carboxy-terminal to the *Eco*RI insert. pPR28 was derived by direct insertion of the mutated *Eco*RI fragment into *Eco*RI partially-restricted plasmid pPR2. All constructs were routinely checked for integrity by both restriction endonuclease analysis and DNA sequencing (Sanger *et al.*, 1977).

The expression and purification of the recombinant proteins as well as their labeling with IAF was as described (Rihs and Peters, 1989).

Mammalian cells, microinjection and laser microscopy

HTC cells were cultured and fused with polyethyleneglycol as described (Rihs and Peters, 1989). Vero cells were obtained from Flow Laboratories (Bonn, Germany) and grown in DMEM medium. Microinjection and microscopic fluorescence measurements were as described (Rihs and Peters, 1989).

Histochemical assay of β-galactosidase

The histochemical X-gal assay for the β-galactosidase activity of the hybrid protein was employed as described (Rihs and Peters, 1989).

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