

# The Cell Cycle–dependent Nuclear Import of v-Jun Is Regulated by Phosphorylation of a Serine Adjacent to the Nuclear Localization Signal

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**Abstract.** Cell cycle–dependent phosphorylation and nuclear import of the tumorigenic transcription factor viral Jun (v-Jun) were investigated in chicken embryo fibroblasts. Nuclear accumulation of v-Jun but not of cellular Jun (c-Jun) is cell cycle dependent, decreasing in G1 and increasing in G2. The cell cycle–dependent regulation of v-Jun was mapped to a single serine residue at position 248 (Ser<sup>248</sup>), adjacent to the nuclear localization signal (NLS). Ser<sup>248</sup> of v-Jun represents an amino acid substitution, replacing cysteine of c-Jun. It was shown by peptidase digestion and immunoprecipitation with antibody to the NLS that v-Jun is phosphorylated at Ser<sup>248</sup> in the cytoplasm but not in the nucleus. This phosphorylation is high in G1 and low in G2. Nuclear accumulation of v-Jun is correlated with un-

derphosphorylation at Ser<sup>248</sup>. The regulation of nuclear import by phosphorylation was also examined using NLS peptides with Ser<sup>248</sup> of v-Jun. Phosphorylation of the serine inhibited nuclear import mediated by the NLS peptide in vivo and in vitro. The protein kinase inhibitors staurosporine and H7 stimulated but the phosphatase inhibitor okadaic acid inhibited nuclear import mediated by the NLS peptide. The cytosolic activity of protein kinases phosphorylating Ser<sup>248</sup> increased in G0 and decreased during cell cycle progression, reaching a minimum in G2, whereas phosphatase activity dephosphorylating Ser<sup>248</sup> was not changed. These results show that nuclear import of v-Jun is negatively regulated by phosphorylation at Ser<sup>248</sup> in the cytoplasm in a cell cycle–dependent manner.

**E**UKARYOTIC cells contain various intracellular membranous organelles that compartmentalize special functions. Specific sorting mechanisms accumulate proteins at their sites of function. In DNA transcription and replication, the movement of proteins from their cytoplasmic site of synthesis to the nuclear interior occurs by translocation across the nuclear pore, a component of the nuclear membrane (for recent review see Dingwall and Laskey, 1992; Forbes, 1992). The nuclear pore complex is a large proteinaceous structure forming a channel with an  $M_r$  of 125,000 kD, consisting of >100 different proteins, including nucleoporins (Davis and Blobel, 1986; Reichelt et al., 1990; Hinshaw et al., 1992). Small molecules freely pass through the pore by diffusion, but larger proteins are specifically selected to enter the nucleus. The nuclear import of proteins is mediated by short amino acid sequences present in most nuclear proteins and known as nuclear localization signals (NLSs)<sup>1</sup> (Kalderon et al., 1984a, b; Garcia-Bustos et al., 1991).

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*Abbreviations used in this paper:* CEF, chicken embryo fibroblast; NLS, nuclear localization signal; PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate.

The nuclear import mechanism is divided into two functional steps (Newmeyer and Forbes, 1988; Richardson et al., 1988). The first consists of the accumulation at the cytoplasmic face of the nuclear pore complex. In this step, the NLS is recognized by a transport mechanism in the cytoplasm or on the nuclear pore. The second step is a translocation into the nuclear interior through the pore channel. This translocation is temperature dependent and requires ATP, as energy (Newmeyer and Forbes, 1988; Richardson et al., 1988), and the GTP-binding protein Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993).

The nuclear import of some proteins is regulated by extracellular stimuli or by the cell cycle. The tumor-suppressor protein p53 accumulates in the nucleus after DNA damage (Fritsche et al., 1993). The transcription factor NF $\kappa$ B is located in the cytoplasm as a complex with I $\kappa$ B, a cytoplasmic anchoring factor (Ghosh and Baltimore, 1990). Extracellular stimuli such as growth factors or tumor-promoting phorbol esters induce dissociation of the complex and translocation of NF $\kappa$ B into the nucleus (Baeuerle and Baltimore, 1988; Ghosh and Baltimore, 1990). In contrast, the nuclear import of the nuclear intermediate filament protein lamin B2 is inhibited by treatment with phorbol ester or by phosphorylation of serine residues adjacent to its NLS (Hennekes et al., 1993). Cyclin D1, which plays an important role in the progression of the cell cycle, is

found in the cytoplasm of G0-arrested cells but translocated to the nucleus in G1 (Baldin et al., 1993). Cyclin B1 and B2 remain in the cytoplasm until the late period of G2 but accumulate in the nucleus in M phase (Pines and Hunter, 1991; Gallant and Nigg, 1992). A budding yeast transcription factor SWI5 locates in the cytoplasm when it is phosphorylated near its NLS in G2 and M but accumulates in the nucleus in G1 concomitant with dephosphorylation (Moll et al., 1991). We previously reported that the nuclear accumulation of the tumorigenic v-Jun is cell cycle regulated (Chida and Vogt, 1992).

Jun is a member of the AP-1 transcription factor family originally found as the product of the oncogene *jun* carried by the avian retrovirus ASV17 (Bohmann et al., 1987; Maki et al., 1987). Viral *jun* (*v-jun*) codes for a fusion protein of  $M_r$  65,000 consisting of viral capsid proteins p19 and  $\Delta p10$  at its amino terminus, followed by the cell-derived *jun* sequences (Maki et al., 1987; Bos et al., 1988; Vogt and Bos, 1990). It accumulates in the nucleus during G2 (Chida and Vogt, 1992). The cellular *jun* product, c-Jun, constantly accumulates in the nucleus, independent of the phase of the cell cycle. The NLS is identical in both v-Jun and c-Jun proteins, consisting of the sequence R<sup>249</sup>KRKL<sup>253</sup> located in the DNA-binding domain (Chida and Vogt, 1992) (Fig. 1). The cell cycle dependence of nuclear translocation was mapped to a single serine residue at position 248 of v-Jun, substituting cysteine of c-Jun. Covalent modification of the serine, for example, phosphorylation, is a possible mechanism for regulating the nuclear import of v-Jun.

In the present study, we found that cell cycle-dependent phosphorylation of Ser<sup>248</sup> inhibited the nuclear import of v-Jun.

## Materials and Methods

### Cell Culture and Synchronization

The avian retroviral expression vector RCAS-VJ0 that contains the entire *v-jun* gene (Bos et al., 1989) was transfected to chicken embryo fibroblasts (CEF). A mutant of v-Jun in which Ser<sup>248</sup> is back mutated to cysteine (GVC100) was similarly expressed by RCAS vector (Chida and Vogt, 1992). The cells were grown in the Ham's F10 medium supplemented with 10% FCS and 4% chicken serum (CLM) at 37°C. Cell synchronization was performed as previously reported (Chida and Vogt, 1992). In brief, cells were arrested in G0 by serum starvation, reducing concentrations of FCS and chicken serum to 1% and 0.4%, respectively, for 3 d. 8 h after changing to the standard serum concentrations of CLM, DNA synthesis started in about 95% of the cells. The G0-arrested cells were exposed to aphidicolin at 10  $\mu$ g/ml in CLM, which resulted in arrest at the G1/S boundary. The cells were released from the block by washing with CLM and then entered S phase, which continued for 6 h. Cytokinesis started 3–4 h after S phase.

### Labeling with <sup>32</sup>P, in Situ Extraction, and Immunoprecipitation

Cells grown in a 35-mm-diam culture dish were incubated for 1 h in 1 mCi/ml of carrier-free <sup>32</sup>P-orthophosphoric acid in phosphate-free DME supplemented with 10% FCS dialyzed against 0.8% NaCl. After washing three times with PBS, the cells were treated with 40  $\mu$ g/ml of saponin in Kern-matrix buffer (Staufenbiel and Deppert, 1984) (20 mM *N*-morpholinoethanesulfonic acid, pH 6.2, 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 10% [vol/vol] glycerol, and 0.1 mM phenylmethylsulfonyl fluoride) for 10 min at 4°C. Using this treatment, a cytoplasmic marker enzyme pyruvate kinase was quantitatively extracted, but nuclear histones were not extracted. Thus, the supernatant was designated as cytoplasmic fraction, and the cells were

washed once with 0.1% Triton X-100 in Kern-matrix buffer. After this extraction, anti-DNA antibody did not permeate in the nuclei, and nuclear immunofluorescence of v-Jun did not decrease, showing the nuclear membrane intact. The cellular residues remaining on the dish were solubilized with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, and 0.1% SDS). This material was designated as nuclear fraction. The total amount of v-Jun in the cytoplasmic and nuclear fractions was almost the same as the amount of whole cellular v-Jun detected by ELISA (Chida and Vogt, 1992), indicating that the fractionation was quantitative.

The cytoplasmic and nuclear fractions were incubated with rabbit anti-Jun antibodies, which had been raised against bacterially produced c-Jun protein (Montecarlo and Vogt, 1993), and with protein A-Sepharose for 1 h at 4°C. After five times washing with RIPA buffer, the protein A-Sepharose-bound phosphoproteins were subjected to SDS-PAGE (Laemmli, 1970), followed by autoradiography.

### Peptidase Digestion

Phosphorylated proteins were electrically eluted from the polyacrylamide gel after SDS-PAGE, dialyzed against water, and concentrated by freeze drying. The isolated phosphoproteins were incubated with 2 mU/ml of asparaginyl endopeptidase (Takara, Kyoto, Japan) in 50 mM sodium acetate (pH 5.0), 1 mM EDTA, 10 mM dithiothreitol, and 1 mM orthovanadate at 37°C for 2 h. The digested peptides were incubated with rabbit antibody raised against a synthetic nuclear localization signal (NLS) peptide representing a part of v-Jun (CA<sup>245</sup>SKSRKRKLERIARL<sup>259</sup>), termed PEP4 (Chida and Vogt, 1992), and protein A-Sepharose in RIPA buffer. The immunoprecipitated peptides were analyzed by SDS-PAGE using a gradient gel with 15–25% of acrylamide. Phospho-amino acid analysis was performed by the method previously described (Wong and Goldberg, 1983).

### Nuclear Import Assay by Microinjection

Synthetic peptides representing NLS sequences of v-Jun are shown in Fig. 5 *a*: D0-6 (CA<sup>245</sup>SKSRKRKRL<sup>253</sup>GG), A2 (CAAKSRKRKLG), A4 (CASKARKRKLGG), A24 (CAAKARKRKLGG), D4-6 (CGGR<sup>249</sup>KRKL<sup>253</sup>GG), and BP-J (CA<sup>234</sup>ERKMRNRRIAASKSRKRKRL<sup>253</sup>GG). The terminal C and GG residues represent an extra cysteine for coupling and glycyl-glycine functioning as spacer, respectively. The NLS peptides were conjugated to rabbit IgG with *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester by the method previously described (Chida and Vogt, 1992). Under these conditions, one molecule of IgG was coupled to an average of six peptide molecules as determined by mobility shift on SDS-PAGE.

Microinjections were performed to the synchronized CEFs grown on cover glass slips (11 × 22 mm) using an automatic microinjector No. 5242 and micromanipulator No. 5171 (Eppendorf Hamburg, Germany) attached to an inverted phase-contrast microscope (Nikon Inc., Tokyo, Japan). The peptide-IgG conjugates at 5 mg/ml in PBS were injected into the cytoplasm of ~400 cells within 15 min at room temperature. The cells were transferred into the CLM medium warmed at 37°C, incubated for 15 min, and then fixed with 4% formaldehyde in PBS. The fixed cells were washed twice with PBS, permeabilized by immersing in methanol for 10 min, and treated with 3% goat serum in PBS for blocking. Injected rabbit IgG was detected by direct immunofluorescence using goat anti-rabbit IgG antibody conjugated to fluorescein isothiocyanate (Zymed Labs, San Francisco, CA). The nuclear import was assayed by counting the cells in which fluorescence of the nucleus was clearly stronger than that of the cytoplasm. The import was expressed as a percentage of the IgG-accumulated nuclei.

### Phosphopeptide Preparation

v-Jun NLS-peptide A2 (5  $\mu$ g/ml) was incubated with protein kinase C (PKC) (1  $\mu$ g/ml) purified from mouse brain (Chida et al., 1986) in a reaction mixture consisting of 20 mM Tris-HCl (pH 7.5), 10 mM ATP, 5 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 64  $\mu$ M phosphatidylserine and 1.3  $\mu$ M diolein at 30°C for 30 min. The resulting peptides were separated by HPLC in an LC-6A apparatus (Shimadzu, Kyoto, Japan) on a reverse phase column Superspher 60 RP-8 (MERCK, Darmstadt, Germany) with linear gradient elution of acetonitrile from 5% to 50%. To remove the phosphate group, the peptide was treated with 0.1 N NaOH at 30°C for 1 h and then analyzed by HPLC. The separated peptides were conjugated to rabbit IgG with *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester. The conjugates were microinjected into the cytoplasm

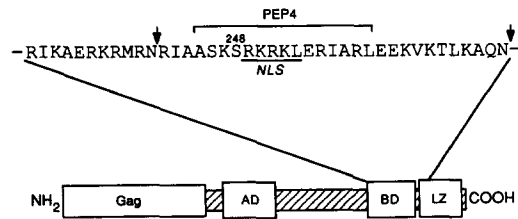
of CEFs, which were treated with 10  $\mu$ M H7, 100 nM staurosporine, and 100 nM okadaic acid. Fifteen minutes after incubation at 37°C, the cells were fixed, and the IgG injected was detected by immunofluorescence.

### In Vitro Nuclear Import Assay

The in vitro nuclear import assay was performed by the method established by Adam et al. (1990). Five synthetic NLS peptides, including D0-6, A2, A4, BP-J (Fig. 5 a) and SV40 T-antigen NLS peptide (CGYGP-KKKRQVGG; Sigma, St. Louis, MO), were conjugated to allophycocyanin (Calbiochem-Novabiochem, La Jolla, CA) with sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL) as previously described (Adam et al., 1990). Approximately six peptides were conjugated to one allophycocyanin molecule as estimated by mobility shift on SDS-PAGE. The normal rat kidney cell line NRK was grown on coverslips in DME supplemented with 10% FCS. The cells were treated with 40  $\mu$ g/ml digitonin in the transport buffer consisting of 20 mM Hepes, (pH 7.3), 110 mM KCl, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml pepstatin at 4°C for 5 min. The permeabilized cells were incubated in the transport buffer supplemented with 100 nM NLS peptide-allophycocyanin conjugate, 1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine phosphokinase MM for 45 min at 30°C. Allophycocyanin accumulated in the nucleus was detected by fluorescence analysis.

### Protein Kinase and Phosphatase Assays

Synchronized CEFs grown in a 10-cm-diameter dish were scraped off with a rubber policeman and suspended in ice-cold extraction buffer consisting of 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 5 mM 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride. After sonication, the cell homogenate was centrifuged at 100,000 *g* for 1 h, then the supernatant was applied to a DE52 (Whatman, Maidstone, England) column. The proteins eluted with 0.5 M NaCl were used as cytoplasmic fraction. The centrifuged pellet was treated with 1% Triton X-100 in extraction buffer, and the solubilized proteins were also applied to a DE52 column. After washing with extraction buffer, proteins were eluted with 0.5 M NaCl and designated as membrane fraction. Protein kinase activity was assayed by measuring incorporation of  $^{32}$ P from [ $\gamma$ - $^{32}$ P]ATP into the A2 NLS-peptide. The reaction mixture consisted of 20 mM Tris-HCl (pH 7.5), 5 mM MgSO<sub>4</sub>, 100  $\mu$ g/ml



**Figure 1.** Location of NLS in v-Jun. v-Jun consists of partial viral Gag sequences representing the p19 and a fragment of the p10 ( $\Delta$ p10) proteins, an activation domain (AD), a basic domain (BD), and a leucine zipper (LZ) dimerization domain. The NLS sequence, RKRKLL, lies next to Ser<sup>248</sup> in the basic domain. Antibody to the NLS was raised against a synthetic peptide representing the PEP4 region. Arrows indicate digestion sites by asparaginyl endopeptidase.

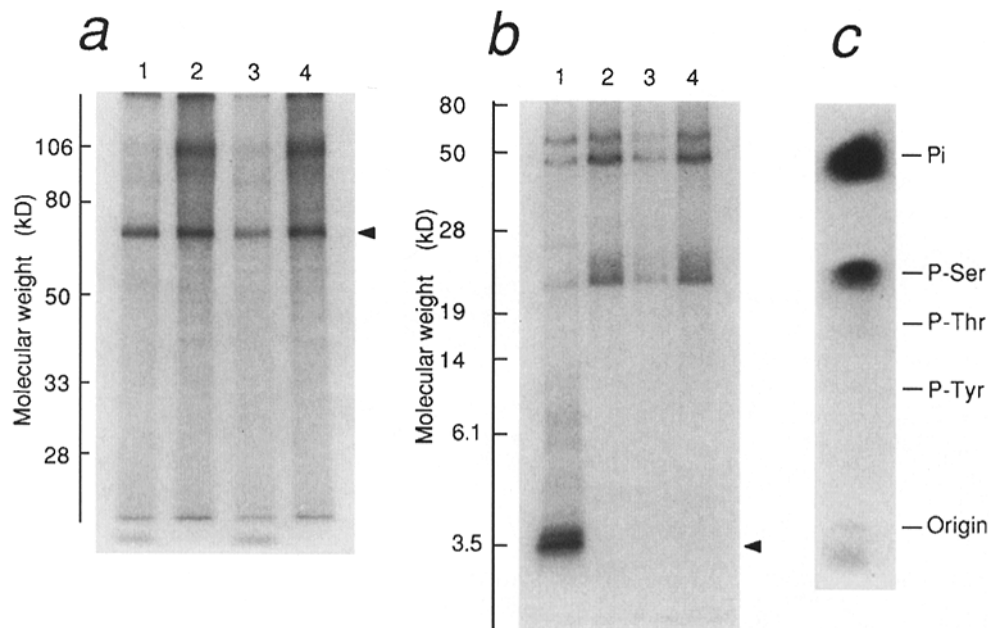
A2 peptide, 10  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP, and 10  $\mu$ l of enzyme preparation in a final volume of 50  $\mu$ l. After incubation for 3 min at 30°C, the reaction mixture was transferred on a piece (1 cm<sup>2</sup>) of anion-exchange filter P81 (Whatman). After washing with 75 mM phosphoric acid, radioactivity on the filter was measured in a liquid scintillation counter.

Phosphatase activity was assayed by measuring release of  $^{32}$ P from  $^{32}$ P-labeled A2 NLS-peptide after phosphorylation with PKC. The reaction mixture consisted of 20 mM Tris-HCl (pH 7.5), 10  $\mu$ g/ml  $^{32}$ P-labeled A2 peptide (30,000 dpm), and 10  $\mu$ l of the enzyme preparation in a final volume of 50  $\mu$ l. After incubation for 10 min at 37°C, P81-binding radioactivity was measured.

## Results

### Cytoplasmic v-Jun Is Phosphorylated at Ser<sup>248</sup>

In a previous study (Chida and Vogt, 1992), the cell cycle-dependent nuclear translocation of v-Jun was mapped to



**Figure 2.** Autoradiographs of phosphorylation at Ser<sup>248</sup> of v-Jun in the cytoplasm. (a) Immunoprecipitation of phosphorylated v-Jun (lanes 1 and 2) and mutant GVC100 containing Cys<sup>248</sup> (lanes 3 and 4) with anti-Jun antibody. Cytoplasmic fractions (lanes 1 and 2); nuclear fractions (lanes 2 and 4). Arrowhead indicates bands of phosphorylated wild-type and mutant v-Jun proteins with *M<sub>r</sub>* 65 kD (10% polyacrylamide gel). (b) Immunoprecipitation by anti-NLS(PEP4) antibody of phosphorylated fragments derived from v-Jun (lanes 1 and 2) and GVC100 (lanes 3 and 4) of cytoplasmic (lanes 1 and 3) and nuclear (lanes 2 and 4) fractions after asparaginyl endopeptidase digestion (15–25% linear-gradient polyacrylamide gel). Prestained molecular weight markers are insulin chain B (3.5 kD), aprotinin (6.1 kD), lysozyme (14 kD), soybean trypsin inhibitor (19 kD), carbonic anhydrase (28 kD), ovalbumin (50 kD), and bovine serum albumin (80 kD). The arrowhead indicates PF3.5, the phosphorylated NLS fragment with an *M<sub>r</sub>* of 3,500. (c) Phospho-amino acid analysis of PF3.5. *Pi*, phosphoric acid; *P-Ser*, phosphoserine; *P-Thr*, phosphothreonine; *P-Tyr*, phosphotyrosine; *Origin*, application site of PF3.5 hydrolysate.

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Ser<sup>248</sup>, which replaces cysteine in the same position of c-Jun (Fig. 1). We hypothesized that phosphorylation of Ser<sup>248</sup> could affect the nuclear transport and therefore studied the phosphorylation of v-Jun in intact cells. For this study, DNA of the expression vector RCAS-VJO containing the entire coding domain of v-jun was transfected into CEF to overproduce and label v-Jun with <sup>32</sup>P. The phosphorylated proteins were immunoprecipitated with antibody to Jun.

As shown in Fig. 2 a, v-Jun with an  $M_r$  of 65 kD was detected in the cytoplasmic and nuclear fractions as a single phosphorylated band. Phosphorylated c-Jun with an  $M_r$  of 40 kD was barely detectable on the original films. Under these conditions, v-Jun is overexpressed by the RCAS vector at least 200-fold over endogenous c-Jun; a mutant of v-Jun, GVC100, in which position 248 is back mutated from serine to cysteine (Chida and Vogt, 1992), was also overexpressed and phosphorylated. GVC100 accumulates in the nucleus at a constant rate similar to c-Jun (Chida and Vogt, 1992). The level of total phosphorylation of cytoplasmic GVC100 appeared to be about one-fifth that of v-Jun in the cytoplasmic fraction. However, the cytoplasmic amount of the GVC100 protein was also reduced to 20%. A difference in total phosphorylation between v-Jun and GVC100 was not detected because both proteins are phosphorylated at numerous sites (~10 sites, including the virus-derived Gag region, as judged by V8 protease and trypsin digestion).

We further examined phosphorylation near the NLS by the combination method of peptidase digestion and immunoprecipitation with anti-NLS antibody. The NLS is located between the two asparagine residues at positions 241 and 270 (Fig. 1). Complete digestion of v-Jun by asparaginyl endopeptidase releases a fragment that consists of 29 amino acid residues with a deduced molecular weight of 3,422. This fragment includes the NLS and can be immunoprecipitated with anti-NLS antibody raised against the peptide (PEP4), encompassing positions 245 to 259 of v-Jun.

Phosphorylated fragments derived from cytoplasmic v-Jun after endopeptidase digestion were separated by SDS-PAGE. They migrated between  $M_r$  3,500 and 55,000 (Fig. 2 b). A phosphorylated fragment with an  $M_r$  of 3,500 (PF3.5) was the major product and probably represents the completely digested fragment that includes the NLS. The other bands with higher molecular weight were partially digested fragments. The phospho-amino acid of PF3.5 was only serine (Fig. 2 c). PF3.5 could be generated only from the cytoplasmic v-Jun but not from the nuclear form. Furthermore, we could not detect PF3.5 in GVC100, the 248-Cys mutant, in either its cytoplasmic or nuclear location. These data show that phosphorylation of PF3.5 is caused by phosphorylation of serine at position 248 (Ser<sup>248</sup>) of v-Jun in the cytoplasm but not in the nucleus.

### Phosphorylation of Ser<sup>248</sup> Is Cell Cycle Dependent

The phosphorylation of Ser<sup>248</sup> in synchronized cells was examined. Phosphorylation of whole v-Jun molecule marginally changed among the cell cycle phases except for G0 (Fig. 3 a). On the other hand, PF3.5 varied during cell cycle progression (Fig. 3 b). PF3.5 was detected in cells arrested in G0 (lane 1). The phosphorylation increased dur-

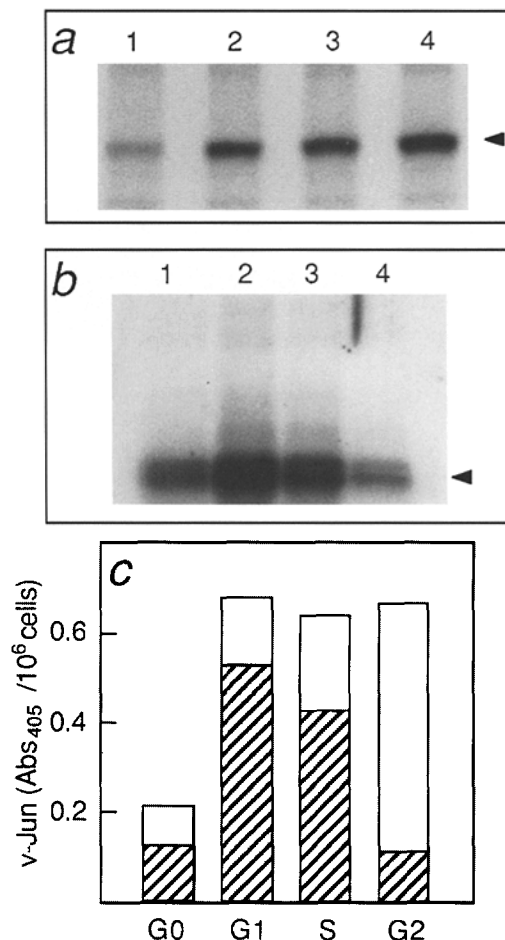
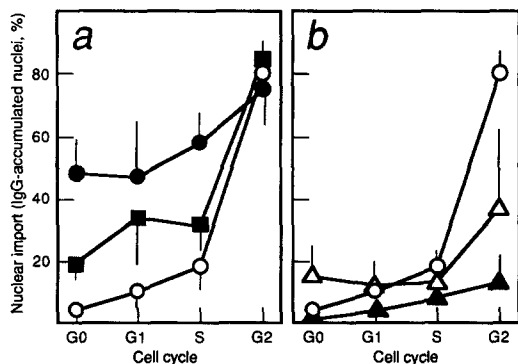


Figure 3. Cell cycle-dependent phosphorylation of Ser<sup>248</sup>. (a) Autoradiograph of the phosphorylated v-Jun immunoprecipitated from synchronized CEF. Cell synchronization was performed by depletion (G0, lane 1) and addition (G1, lane 2) of serum, and aphidicolin block and release (S and G2, lanes 3 and 4). The arrowhead indicates v-Jun with  $M_r$  65 kD. (b) Autoradiograph of PF3.5 (arrowhead). Lane numbers correspond to those in Fig. 3 a. (c) Change of intracellular distribution of v-Jun. Quantities of v-Jun in the nuclei and cytoplasm were assayed by ELISA and shown as open and shaded columns, respectively.

ing G0–G1 transition, reaching a maximum in G1 (lane 2), slightly decreasing in S (lane 3), and reaching a minimum in G2 (lane 4). Although the phosphorylation changed, the total amount of v-Jun remained virtually constant during cell cycle progression, except for the G0, when amounts of Jun protein were at about 30% of the maximum (Fig. 3 c). The phosphorylation at Ser<sup>248</sup> was correlated with the amounts of v-Jun remaining in the cytoplasm in a cell cycle-dependent fashion.

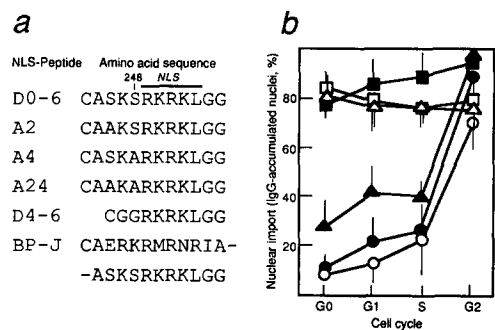
### Protein Kinases and Phosphatases Regulate the Cell Cycle-dependent Nuclear Import of v-Jun

We examined effects of inhibitors of protein kinases and of phosphoprotein phosphatases on nuclear import mediated by synthetic NLS peptide. The NLS peptide D0-6 mediated translocation of a carrier protein, rabbit IgG, into the nucleus in a cell cycle-dependent manner (Fig. 4)



**Figure 4.** Stimulation by protein kinase inhibitors and inhibition by a phosphoprotein phosphatase inhibitor of cell cycle-dependent nuclear import. A conjugate of the NLS peptide D0-6 and IgG was microinjected into the cytoplasm of synchronized CEF. The cells were incubated with protein kinase inhibitors (a) H7 (10  $\mu$ M, closed squares) or staurosporine (100 nM, closed circles), and (b) phosphatase inhibitor okadaic acid (0.1  $\mu$ M, open triangles); 1  $\mu$ M, closed triangles) at 37°C for 15 min. Nontreated controls, open circles (a and b). Nuclei-accumulating IgG were counted. Bars, SD of the three measurements; the absence of bars indicates that SD is within the symbol.

(Chida and Vogt, 1992). The rate of nuclear import was low in G0, G1, and S but high in G2. Treatment with protein kinase inhibitors H7 (10  $\mu$ M) or staurosporine (100 nM) stimulated transport in the G0, G1, and S phases (Fig. 4 a). On the other hand, okadaic acid, an inhibitor of phosphoprotein phosphatases, blocked the nuclear import in G2 (Fig. 4 b). In contrast, the cell cycle-independent NLS peptide D4-6 (Fig. 5 a) and the SV40 T-antigen NLS peptide (CGYGPKKKRKVGG) were not affected in their nuclear import by these inhibitors (data not shown), indicating that the nuclear translocation mechanism itself was not nonspecifically disturbed by the inhibitors. The results suggest that protein kinase(s) and phosphatase(s) regulate



**Figure 5.** Involvement of Ser<sup>248</sup> in cell cycle-dependent nuclear import. (a) Sequence of alanine-substituted NLS peptides. (b) Cell cycle-dependent nuclear import mediated by D0-6 (open circles), A2 (closed circles), and BP-J (closed triangles) NLS-peptides with Ser<sup>248</sup>. The conjugate of the NLS peptide and IgG was microinjected into the cytoplasm of synchronized CEF. After incubation at 37°C for 15 min, nuclei that had accumulated IgG were counted. NLS peptides without Ser<sup>248</sup> are A4 (open squares), A24 (closed squares), and D4-6 (open triangles). Bars, SD of the three measurements; the absence of bars indicates SD is within the symbol.

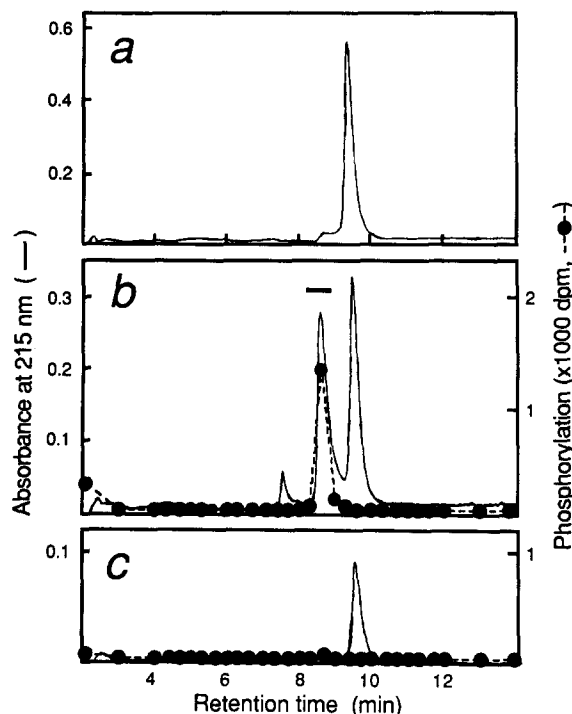
the cell cycle-dependent nuclear import mediated by the D0-6 NLS-peptide.

### Cell Cycle-Dependent Nuclear Import of $\nu$ -Jun Requires Ser<sup>248</sup>

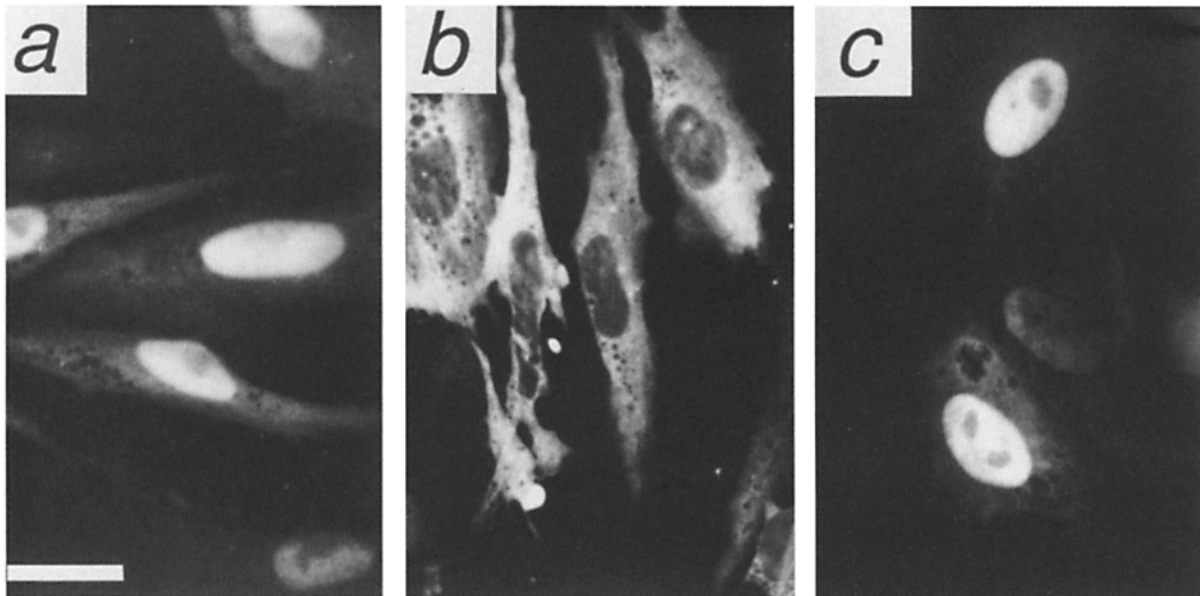
We focused on the importance of the serine residue in the D0-6 NLS-peptide for the cell cycle-dependent regulation and synthesized other NLS peptides in which serine at position 246 or 248 was substituted by alanine (Fig. 5 a). All these peptides mediated nuclear import (Fig. 5 b). The A2 peptide with Ala<sup>246</sup> and Ser<sup>248</sup> showed cell cycle dependence in nuclear import similar to the D0-6 peptide. The longer BP-J peptide, including Ser<sup>248</sup> and bipartite NLS structure (Robbins et al., 1991; Mikaélian et al., 1993), also showed the dependence. However, the A4 peptide with Ser<sup>246</sup> and Ala<sup>248</sup>, and the A24 peptide with two alanine residues at positions 246 and 248, mediated import in a cell cycle-independent manner as well as with D4-6, which contained only the minimum NLS (Fig. 5 a) (Chida and Vogt, 1992). The presence of Ser<sup>248</sup> was correlated with cell cycle dependence in nuclear import.

### Phosphorylation of Ser<sup>248</sup> Inhibits Nuclear Import

We examined six purified serine/threonine protein kinases for their ability to phosphorylate the serine residue of the A2 NLS-peptide, including PKC, the catalytic subunit of protein kinase A, cyclin-dependent kinase p34<sup>cdc2</sup>, casein



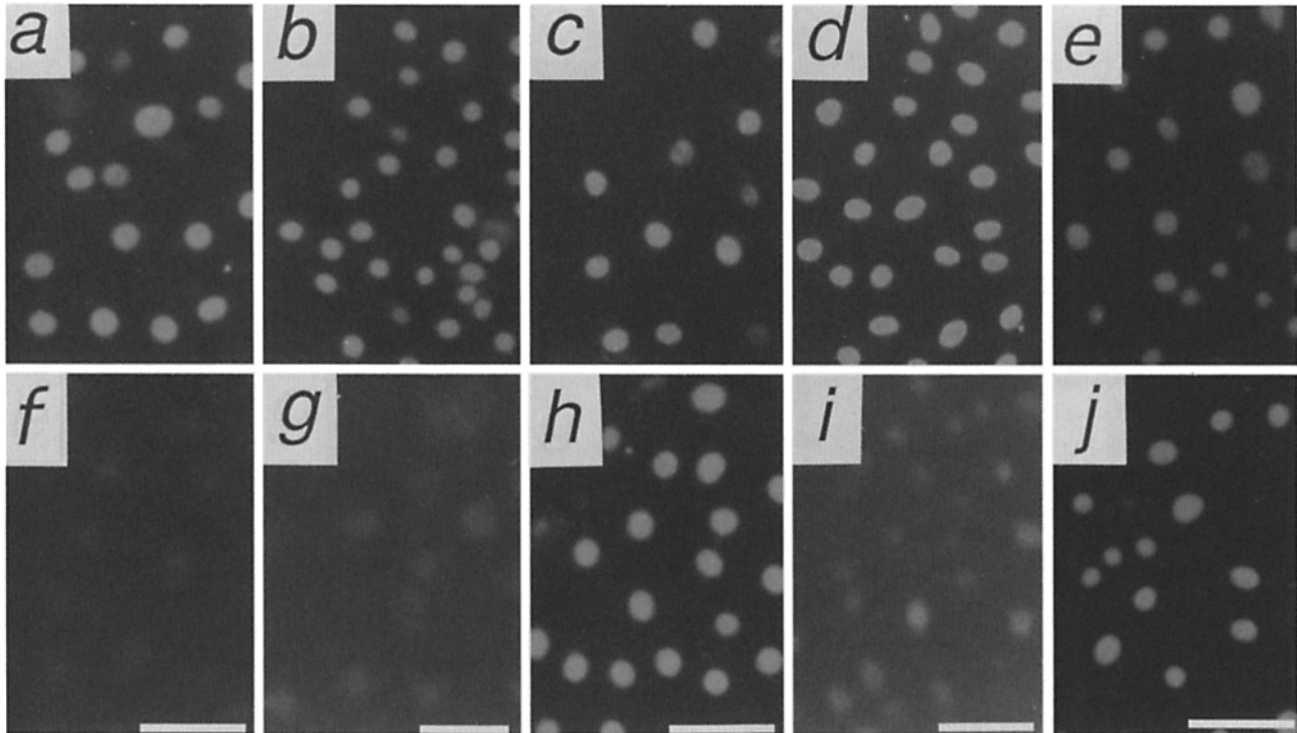
**Figure 6.** Isolation of phosphorylated A2 NLS-peptide by HPLC. The A2 peptide (a) was phosphorylated by PKC, and the resulting phospho-peptide (b) was separated by HPLC using a C8 reverse phase column. The fraction with the phosphorylated peptide (closed circle) was recovered (b, bar) at 8.5 min of retention time and treated with 0.1 N NaOH to remove the phosphate group (c).



**Figure 7.** Inhibition of nuclear import by phosphorylation at Ser<sup>248</sup> in vivo. Nontreated (*a*), phosphorylated (*b*), and dephosphorylated (*c*) A2 NLS-peptides purified by HPLC were conjugated to IgG and microinjected into the cytoplasm of CEF treated with 10  $\mu$ M H7, 100 nM staurosporine, and 100 nM okadaic acid. After incubation at 37°C for 15 min, the conjugate was detected by immunofluorescence. Bar, 20  $\mu$ m.

kinase I and II, and microtubule-associated protein kinase. Among the protein kinases used, PKC efficiently phosphorylated the A2 NLS-peptide in a cell free-system (data not shown). The other protein kinases phosphorylated the A2 peptide only poorly or not at all.

Therefore, we used PKC to phosphorylate the serine residue of the A2 NLS-peptide and separated the phosphorylated form by HPLC (Fig. 6). The A2 peptide was retained for 9.5 min under the conditions of the HPLC run (Fig. 6 *a*); treatment with PKC caused the appearance of a



**Figure 8.** In vitro nuclear import. Allophycocyanin conjugates with NLS peptides D0-6 (*a* and *f*), A2 (*b* and *g*), A4 (*c* and *h*), BP-J (*d* and *i*), and SV40 T-antigen (*e* and *j*) were treated with PKC (*f-j*) or remained untreated (*a-e*). The conjugates were incubated at 37°C for 30 min with digitonin-treated NRK cells. Fluorescence accumulating in the nucleus was determined. Bars, 50  $\mu$ m.

new and faster-moving peak with 8.5 min of retention time containing the  $^{32}\text{P}$  label (Fig. 6 b). Treatment of the phosphopeptide with 0.1 N sodium hydroxide released the phosphate group and reversed the HPLC retention time to that of the unphosphorylated A2 peptide (Fig. 6 c).

The phosphorylated A2 peptide was covalently conjugated to rabbit IgG and microinjected into the cytoplasm of CEF treated with H7, staurosporine, and okadaic acid to prevent further phosphorylation and dephosphorylation of the serine residue in the injected cells (Fig. 7). The complex of IgG and phosphorylated A2 NLS-peptide remained in the cytoplasm of the injected cells (Fig. 7 b). In contrast, unphosphorylated A2 peptide or the dephosphorylated form was able to mediate nuclear import. Phosphorylation of Ser<sup>248</sup> inhibited the nuclear import activity of the NLS peptide (Fig. 7, a and c).

### *Ser<sup>248</sup> Is Necessary for the Phosphorylation-dependent Inhibition of Nuclear Import In Vitro*

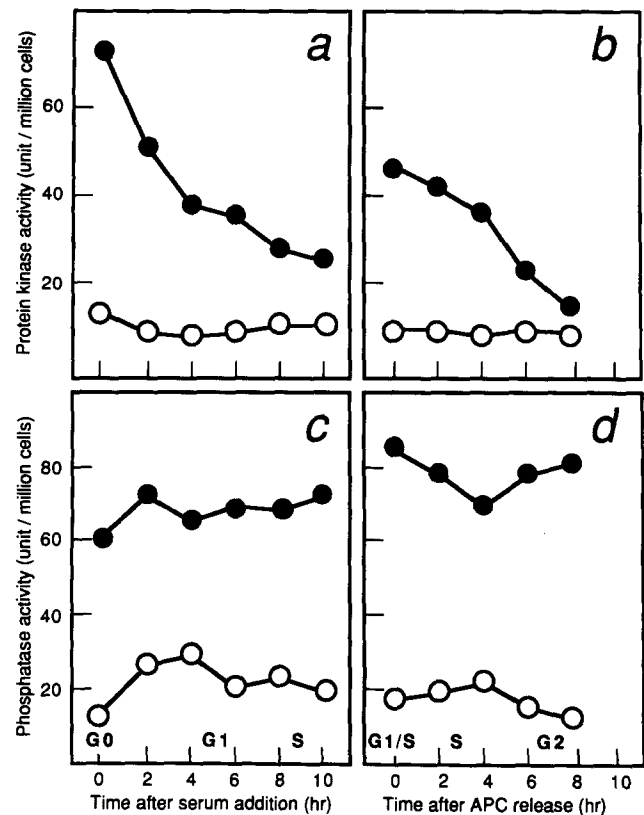
We further examined effects of phosphorylation on nuclear import on the in vitro system originally developed by Adam et al. (1990). Permeabilized NRK cells and fluorescent allophycocyanine conjugated to various NLS peptides were incubated, and fluorescence accumulating in the nucleus was determined. All of the NLS peptides used mediated nuclear import in vitro (Fig. 8). Pretreatment with PKC inhibited the nuclear import by D0-6, A2, and BP-J peptides: they include the Ser<sup>248</sup> of v-Jun and mediate cell cycle-dependent import in intact cells (Fig. 5). However, PKC did not inhibit the import mediated by the A4 peptide with Ala<sup>248</sup> or with the SV40 T-antigen NLS peptide. The presence of Ser<sup>248</sup> was therefore correlated with inhibition of in vitro nuclear import after PKC treatment.

### *Cytosolic Kinase Activity for the Jun A2 Peptide Is Cell Cycle Dependent*

We assayed the enzymatic activities controlling the phosphorylation state of the A2 NLS-peptide. Protein kinase activity in the cytosol fraction of synchronized CEF increased in G0 and decreased during cell cycle progression, reaching a minimum in G2 (Fig. 9, a and b). On the other hand, the protein kinase activity in the membrane fraction was less than that in the cytosol and remained constant during the cell cycle. Phosphatase activity was measured using  $^{32}\text{P}$ -labeled A2 peptide as substrate; it did not change during the cell cycle in either cytosol or membrane fractions (Fig. 9, c and d). These results suggest that phosphorylation of Ser<sup>248</sup> in v-Jun is caused by cell cycle-controlled protein kinase(s) in the cytoplasm.

### *Discussion*

In the present study, we have shown a relationship between phosphorylation of v-Jun and its retention in the cytoplasm of intact cells. We have identified Ser<sup>248</sup> as the critical site; its phosphorylation varies during cell cycle phases, reaching a maximum in G1 and a minimum in G2. We found that NLS peptides that contain Ser<sup>248</sup> are able to mediate G2-specific nuclear import and that phosphorylation of this serine residue blocks nuclear import.



**Figure 9.** Protein kinase and phosphoprotein phosphatase activities in synchronized CEF. Cytosol (closed circles) and membrane (open circles) fractions were prepared from CEF synchronized by serum depletion (a and c) and aphidicolin blocking (b and d). Protein kinase activity was measured using the A2 NLS-peptide as a substrate (a and b). One unit of the protein kinase is defined as incorporation of 1 pmol of phosphate per minute at 30°C from ATP into A2 peptide. Phosphoprotein phosphatases were measured using phosphorylated A2 peptide as a substrate (c and d). One unit of phosphatase is defined as release of 1% of phosphate per minute at 30°C from the phosphorylated A2 peptide. The SD of the three measurements was less than 8% of the mean value.

The phosphorylation state of a protein is determined by a balance of protein kinase and phosphoprotein phosphatase activities. A soluble and cell cycle-dependent kinase may be responsible for the periodic phosphorylation of v-Jun. There are several protein kinases phosphorylating c-Jun in intact cells. Casein kinase II phosphorylates threonine at position 210 and serine at 222 (Lin et al., 1992), glycogen synthase kinase 3 targets threonine at 218 and two serine residues at 222 and 228 (Boyle et al., 1991), and a proline-directed kinase JNK1 or SAPK phosphorylates two serine residues at 59 and 69 (Dérillard et al., 1994; Kyriakis et al., 1994). (Numbers refer to residues in the chicken c-Jun protein; Nishimura and Vogt, 1988). Furthermore, c-Jun is phosphorylated at tyrosine residues after treatment of cells with insulin-like growth factor 1 (Oemar et al., 1991). These phosphorylations may affect functions of c-Jun in transcription, including DNA binding and the redox state at Cys<sup>248</sup> (Abate et al., 1990). However, the protein kinase that phosphorylates Ser<sup>248</sup> of v-Jun in living cells has not yet been identified.



In a cell free-system, purified PKC effectively phosphorylated the v-Jun NLS peptides A2 and D0-6; both include consensus sequences for PKC phosphorylation (Kennelly and Krebs, 1991). However, PKC activity did not change during cell cycle progression in CEF (data not shown). Furthermore, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), which strongly activates most isoforms of PKC, did not affect the nuclear accumulation of v-Jun or the nuclear import mediated by cell cycle-dependent Jun-derived NLS peptides (data not shown). PKC forms a family consisting of at least eleven isoforms that are separated into two groups according to their response to TPA (for review see Nishizuka, 1992). The TPA-activatable PKC isoforms, including  $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ , appear not responsible for the regulation of nuclear import of v-Jun. The possibility remains that the TPA-insensitive PKC isoforms  $\zeta$  or  $\lambda$  are cell cycle-controlled and regulate the nuclear translocation of v-Jun.

Many NLS domains have been identified in various nuclear proteins of virus, yeast, amphibian, avian, and mammalian origin (for review see Garcia-Bustos et al., 1991). Although a universal consensus sequence for nuclear localization has not been established, the known signals were divided into two major classes (Robbins et al., 1991). The first class consists of a stretch of three to five basic residues, typified by the SV40 T-antigen NLS (PKKKRKV) (Kalderon et al., 1984a, b). The second class, known as bipartite NLS, consists of two basic amino acids at the NH<sub>2</sub>-terminal side, an intervening spacer of 10 amino acids, and a stretch of three or four basic amino acids at the COOH-terminal side; an example is the nucleoplasmin NLS (KRP-AATKKAGQAKKKK; underlines showing bipartite region) (Dingwall et al., 1988; Robbins et al., 1991). The bipartite NLS structure is found in many nuclear proteins, including all members of the Jun family. Efficient nuclear accumulation of c-Jun in HeLa cells has been reported to require the complete bipartite NLS structure (Mikaélian et al., 1993). The bipartite NLS of Jun may play a role in import in some cell types, but a synthetic peptide encompassing only the carboxyl part was able to mediate nuclear import of an unrelated protein with the same efficiency as the T-antigen NLS (Chida and Vogt, 1992).

The regulation of nuclear import mediated by NLS phosphorylation was first demonstrated on SV40 T-antigen (Rihs and Peters, 1989). Phosphorylation by p34<sup>cdc2</sup> or casein kinase II inhibits nuclear import mediated by the T-antigen NLS (Jans et al., 1991; Rihs et al., 1991). The transcription factor SWI5 is phosphorylated at an internal serine residue within the bipartite NLS, correlating with retention in the cytoplasm of budding yeast (Moll et al., 1991). Lamin B2, with a simple NLS similar to that of the T-antigen, is phosphorylated after treatment of cells with TPA and by purified PKC in a cell free-system (Kasahara et al., 1991; Hennekes et al., 1993). The treatment of lamin B2 with PKC $\alpha$  inhibits nuclear import; the phosphorylation sites are two serine residues located three and four positions NH<sub>2</sub>-terminal to the NLS (Hennekes et al., 1993), similar to v-Jun. Phosphorylation near a simple NLS or in bipartite NLS may therefore negatively regulate transport into the nucleus.

One possible reason why phosphorylation near the NLS interferes with targeting the protein to the nucleus may

have to do with electrostatic interaction. The phospho-group could neutralize a positive charge of the NLS and thus prevent interaction with an NLS recognition molecule. A second possible explanation derives from a conformational change in NLS structure. The presence of a phospho-group may prevent the recognition molecule from correctly and tightly binding to the NLS, and consequently nuclear import would not take place. Another possibility depends on the regulation of a bipartite NLS. Phosphorylation within the intervening spacer may interfere with the nuclear import machinery. There are reports on proteins that bind to NLSs (Adam et al., 1989; Yamasaki et al., 1989; Adam and Gerace, 1991; Imamoto et al., 1992; Adam and Adam, 1994; Görlich et al., 1994). However, it is still unclear what molecule initially interacts with the NLS to initiate nuclear import in the living cell. Adam and Adam (1994) and Adam and Gerace (1991) described cytosolic 54-, 56-, and 97-kD proteins that are required for nuclear import in an in vitro transport assay. Görlich et al. (1994) reported that a cytoplasmic 60 kD protein named importin promotes NLS-dependent binding of karyophilic proteins to the nuclear envelope. The 70-kD heat shock protein hsp70 and its cognate protein hsc70 are required for nuclear import (Imamoto et al., 1992; Yang and De-Franco, 1994). These proteins or other unknown factors might bind to the v-Jun NLS and effect nuclear import, but they may fail to bind if Ser<sup>248</sup> is phosphorylated.

Our previous work has shown that c-Jun does not induce tumors in the animal, while v-Jun is tumorigenic in vivo (Wong et al., 1992). The Cys-Ser mutation at position 248 makes c-Jun tumorigenic (Morgan et al., 1994). However, the change is only one of several mutations found in v-Jun that can all independently activate the tumorigenic potential of c-Jun (Morgan et al., 1994). Although the altered regulation in nuclear entry of v-Jun may be related to its tumorigenicity, further studies are needed for understanding tumor formation by v-Jun.

In conclusion, our results demonstrate that nuclear import of v-Jun is sensitive to cell cycle-controlled changes in the state of phosphorylation of a serine residue next to the NLS. These observations suggest that regulation of nuclear import by phosphorylation may represent an important mechanism in the periodic and temporal sorting of nuclear proteins.

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