Control over the nuclear import of transcription factors (TFs) represents a level of gene regulation integral to cellular processes such as differentiation and transformation. The Drosophila TF Dorsal shares with other rel TF family members the fact that it contains a phosphorylation site for the cAMP-dependent protein kinase (PKA) 22 amino acids N-terminal to the nuclear localization signal (NLS) at amino acids 335–340. This study examines for the first time the nuclear import kinetics of Dorsal fusion proteins in rat hepatoma cells in vivo and in vitro. Nuclear uptake was found to be not only NLS-dependent, but also strongly dependent on the PKA site, whereby substitution of Ser^312 by either Ala or Glu using site-directed mutagenesis severely reduced nuclear accumulation. Exogenous cAMP or PKA catalytic subunit significantly enhanced the nuclear import of wild-type proteins both in vivo and in vitro. Using a direct binding assay, the molecular basis of PKA site enhancement of Dorsal fusion protein nuclear import was determined to be PKA site-mediated modulation of NLS recognition by the importin 58/97 complex. The physiological relevance of these results is supported by the observation that Drosophila embryos expressing PKA site Dorsal mutant variants were impaired in development. We conclude that the Dorsal NLS and PKA site constitute a phosphorylation-regulated NLS essential to Dorsal function and able to function in heterologous mammalian cell systems, where phosphorylation modulates the affinity of NLS recognition by importin.

Precisely scheduled nuclear import of transcription factors (TFs)\(^1\) is a key factor in eukaryotic cell function (1–3). While proteins such as histones appear to be constitutively targeted to the nucleus, TFs such as those of the rel family (1, 4–7), the nuclear factor of activated T-cells (8), SWI5 from yeast (9, 10), and the cytokine responsive signal transducers and activators of transcription (STATs) (11, 12) are translocated to the nucleus only under specific conditions, being otherwise cytoplasmic and thereby directly accessible to cytoplasmic signal-transducing systems (13). The fact that nuclear translocation of many TFs and oncogene products accompanies changes in the differentiation or metabolic state of eukaryotic cells underlines the fact that nuclear protein import is a key control point in the regulation of gene expression (2, 3).

Proteins larger than 45 kDa require a nuclear localization sequence (NLS) (2, 3) in order to be targeted to the nucleus. We and others have shown that phosphorylation in the vicinity of NLSs plays a central role in regulating NLS-dependent nuclear protein import (7–21). The modular sequence motifs able to confer regulated nuclear protein import on heterologous proteins, called phosphorylation-regulated NLSs (prNLSs) (2, 3), have been identified for a number of proteins, including the CcN motif of the simian virus SV40 large tumor antigen (T-ag) whose transport is regulated by dual phosphorylation by protein kinase CK2 (CK2) and the cyclin-dependent kinase cdc2 (13–15), and the cell cycle-dependent NLS of the yeast TF SWI5 (9, 10). Significantly in the case of SWI5, cyclin-dependent kinase site-mediated inhibition of SWI5 nuclear transport functions in higher eukaryotes (10). A variety of kinases are known to modulate nuclear protein import in response to specific hormonal or other cellular signals regulating their activity (2, 3). In the case of the T-ag CcN motif, we have shown that substitution of one kinase site by a consensus site for another can alter the cellular signals able to regulate the nuclear import of proteins carrying the modified prNLS (19).

Although a number of prNLSs have been defined, the mechanistic basis of prNLS-mediated regulation of nuclear transport is largely unclear. Our recent work with respect to CK2 enhancement of T-ag nuclear import indicates that phosphorylation facilitates recognition of the T-ag NLS by the NLS-binding importin subunits (22). We were interested in measuring the kinetics of nuclear accumulation of the rel family member Dorsal, the Drosophila melanogaster morphogen whose graded nuclear translocation is integral in determining dorsal-ventral polarity during embryogenesis (4). Modifying genes that regulate Dorsal nuclear accumulation include the transmembrane receptor protein Toll, the Raf family kinase Pelle, the protein Tube, which may have a chaperone function with respect to Dorsal or Pelle, and the cytoplasmic retention factor Cactus (23–27), which binds Dorsal and prevents its nuclear localization although not through direct binding to the Dorsal NLS (27). Results using a number of experimental sys-

\(^1\) The abbreviations used are: TF, transcription factor; NLS, nuclear localization sequence; prNLS, phosphorylation-regulated NLS; PKA, cAMP-dependent protein kinase; T-ag, SV40 large tumor-antigen; CK2, protein kinase CK2 (casein kinase II); CLSM, confocal laser scanning microscopy; ELISA, enzyme-linked immunosorbent assay; GTP\_\_S, guanosine 5′-O-(thiotriphosphate).
tems have suggested the involvement of phosphorylation in regulating Dorsal nuclear localization (23, 28–32). In identical fashion to other rel family members, Dorsal possesses a consen- sus site for PKA 22 amino acids N-terminal to a 6- amino acid NLS within the ~300-amino acid rel homology domain (see Fig. 1), where the enhancing role of PKA in terms of nuclear localization has been qualitatively described, using transfec-
tion systems for Dorsal (23) and c-rel (7), and implicated for NF-κB p65 (5, 6, 23).

In this study we define the Dorsal prNLS kinetically by quantifying the nuclear uptake of β-galactosidase fusion proteins at the single cell level both in vivo and in vitro in the HTC rat hepatoma line using confocal laser scanning microscopy (CLSM) (10, 13–15, 19). While Dorsal fusion protein nuclear uptake is NLS-dependent, it is also strongly dependent on the PKA site, whereby substitution of Ser312 by either Ala or Glu using site-directed mutagenesis essentially abolishes nuclear accumulation. Exogenous addition of CAMP or PKA catalytic (C-) subunit enhances nuclear import of the wild-type Dorsal proteins. Results using an ELISA-based binding assay indicate that the mechanistic basis of the PKA site modulation of Dorsal fusion protein nuclear import is PKA-site enhancement of the binding interaction between the NLS-binding importin 58/97 complex and the Dorsal NLS. The fact that Drosophila embryos expressing PKA site and NLS Dorsal mutant variants show impaired development and fail to hatch supports the physio-
logical relevance of the results. We conclude that the Dorsal NLS and PKA site constitute a prNLS essential to Dorsal function, and that function in heterologous mammalian cell systems, where phosphorylation regulates interaction with import

PORTIN 58/97.

Materials and Methods

Chemicals and Reagents—PKA (EC 2.7.1.37) catalytic (C-) subunit (bovine heart) was from Sigma; other reagents were from the sources previously described (10, 15, 19, 22).

Cell Culture—Cells of the HTC rat hepatoma tissue culture (a derivative of Morris hepatoma 7228C) line were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum as described previously (10, 15, 19).

β-Galactosidase Fusion Proteins—Plasmids expressing the Dorsal β-galactosidase fusion proteins were derived by standard recombinant DNA technology. Inserts encoding Dorsal amino acids 1–678 (D1 con-
struct, full-length Dorsal), 1–346 (D2 construct, encoding the rel homol-
ogy domain), and 297–346 (D3 construct, encoding the PKA site at Ser312 together with the NLS at amino acids 335–340), were derived using a polymerase chain reaction and ligated into the NcoI site of a derivative of plasmid vector pPR2 (14) in which the oligonucleotide 5′-GCCATGGTTGTA-3′ was inserted into the SmaI site. The D3 con-
struct containing the NLS-deficient mutant (Thr337Glu339), as well as plasmids encoding the PKA site Ser312 substitutions by Ala or Glu (see Fig. 1) were derived by oligonucleotide site-directed mutagenesis of the wild-type Dorsal β-galactosidase fusion protein-expressing constructs using the Amersham Pharmacia Biotech U.S.E. mutagenesis kit. The T-ag-CcN-β-galactosidase fusion protein used as a control contains T-ag amino acids 111–135, encompassing the CcN motif (including CK2 and cyclin-dependent kinase phosphorylation sites and NLS) fused N-termi-

nal to the Escherichia coli β-galactosidase enzyme sequence (amino acids 9–1023) (13, 14).

In Vivo Nuclear Transport Assay—Analysis of nuclear import kinetics at the single cell level in microinjected HTC cells using CLSM (Bio-Rad MRC-500) was as described previously (10, 19, 22). HTC cells were fused with polyethylene glycol about 1 h prior to microinjection to produce polykaryons (13–15, 19). Image analysis of CLSM files, using the Macintosh NIH Image 1.49 public domain software, and curve fitting was performed as described elsewhere (19, 22).

In Vivo Nuclear Transport Assay—Analysis of nuclear import kinetics at the single cell level using mechanically perforated HTC cells in conjunction with CLSM was as described previously (13, 19, 34). NLS-
dependent nuclear protein import can be reconstituted in this system through the exogenous addition of cytosolic extract (untreated reticu-
locyte lysate, Promega catalog no. L415A), an ATP-regenerating system (0.125 mg/ml creatine phosphokinase, 30 μm creatine phosphate, 2 μM ADP, and 5 mM transport substrate Mg2+), and a cytosolic extract of Drosophila embryo (inclusion body of labeled fusion protein). Image analysis and curve-fitting were performed as for in vivo assays.

Where indicated, proteins were either coinjected with CAMP (250 μM in the pipette) in the case of the in vivo experiments, or 25 μM cAMP, 2.5 μg PKA C-subunit inhibitor peptide PRI 5–24, and 400 picomolar units of PKA C-subunit (a gift from Dr. J. McKnight) into the cytosol in the case of in vitro experiments (19). In experiments where the dependence of transport on the GTP-binding protein Ran/TC4 was tested, cytosolic extract was treated with 850 μM GTP·S (nonhydrolyzable GTP analog) for 5 min at room temperature, prior to use in the in vitro assay (34). The lectin wheat germ agglutinin, which impairs nuclear pore complex function, was used at 240 μg/ml.

In Vitro Phosphorylation—In vitro phosphorylation of fusion proteins by PKA C-subunit was analyzed quantitatively by determination of the stoichiometry of phosphorylation as described previously (13, 19, 22).

Expression of Mouse Importin 58 and 97 Fusion Proteins—Mouse importin 58- and 97-glutathione S-transferase fusion proteins were expressed and purified as described previously (22, 24), with glutathione S-transferase-free mouse importin 58 prepared by subsequent throm-
bin cleavage (22).

ELISA-based Binding to Quantitate NLS Recognition—Binding of importin subunits to β-galactosidase fusion proteins was quantitated using an ELISA as described previously (22, 34). Briefly, fusion proteins (0.5 μg/well) were coated overnight at 4°C in 96-well microtiterplates (Nunc). After blocking, appropriate dilutions of mouse importin 58- and 97-glutathione S-transferase or precomplexed importin 58/97-glutathione S-transferase complex were then added to the wells and incubated for 16 h at 4°C. Bound importin was detected using rabbit anti-glutathione S-transferase and goat anti-rabbit IgG alkaline phosphatase-conju-
gated antibodies (Sigma) and the colorimetric substrate p-nitrophenyl phosphate. A405 was followed with time using a plate reader (Molecular Devices), with values corrected by subtracting both the absorbance at 0 min and the absorbance of wells incubated without importin 58- or 58/97-glutathione S-transferase complexes. Correction was made for differ-
ences in coating efficiency as described previously using a parallel β-galactosidase ELISA (22, 34).

Production of Dorsal Mutant Transgenic Flies—Plasmid pBP-dorsal is a derivative of pSP64 (35) that carries the wild-type dorsal cDNA (38) chimerically fused downstream of the Xenopus globin leader, which is known to direct efficient translational initiation in Drosophila embryos (37). Site-directed mutagenesis (see above) was used to change the PKA site Ser312 to Ala and to Glu, and to create the NLS-deficient double mutant (Lys337Thr339Gln340Val341) to Glu in pBP-dorsal to generate dorsal mutants exactly comparable to those described above for the bacterially expressed Dorsal fusion proteins. For each dorsal construct, about 10 eggs were set up to carryout PCR screening of the translation signals and modified dorsal cDNA was excised and introduced in the appropriate orientation plasmid pCasperbcgβIII, a P-element-derived vector (37) carrying the promoter region of the bcd gene (38), which directs transcription in the female germ line. As a wild-type control, a dorsal cDNA carrying the Met-His-N-terminal epitope tag, cloned in bP4-dorsal, was similarly introduced into pCasperbcgβIII. The epitope tag has no effect on Dorsal protein function. The P-element vectors carrying the dorsal cassettes were then introduced into the Drosophila genome through conventional transformation methods (39).

To determine their ability to substitute for the wild-type dorsal gene in defining embryonic dorsal-ventral pattern, the mutant transgenes were crossed into females of the genotype w; M/dl1. The presence of the w + eye marker allowed identification of females carrying the trans-
gen transgenes by their colored eyes. Genetically, the dl1 allele behaves like a null mutation; Dorsal protein is not present in embryos produced by dl/dl1 females (see also Fig. 7B). dorsal mutant females carrying the various dorsal mutant transgenes were placed on yeast and allowed to lay eggs. Cyticul prepara-
tions of the embryos produced by these females were made 72 h after egg deposition (40).

Multiple transgenic lines were obtained and analyzed for each construct; significant differences in expression are associated with differ-
ent chromosomal sites of insertion. Although the level of phenotypic rescue was relatively constant for each insert identified for a particular dorsal transgene from line to line, there were differences in the level of rescue, and analysis was restricted to those insertions that provided the highest level of rescue of the dorsal mutant phenotype. About a third of
transgenic inserts obtained for a particular construct were found to provide the highest level of observable rescue (see “Results”); two of six stocks carrying p(w-, bed-dorsal, NLS mutant) led to the formation of embryos producing Filzkörper material, three of 10 stocks carrying either p(w-, bed-dorsal, Ala312) or p(w-, bed-dorsal, Glu312) led to the formation of embryos producing ventral denticles as well as Filzkörper material, and one third of transgenic lines carrying the wild-type dorsal gene under the transcriptional control of the bcd promoter produced hatching embryos when present in single copy in females that were otherwise mutant for dorsal. Consistent with one third of transgenic lines producing a relatively comparable high level of expression, whole mounts of embryos from the best rescued females carrying wild-type dorsal, dorsal, Ala312, and dorsal NLS mutant exhibited similar levels of staining for Dorsal protein (Fig. 7).

**Immunostaining—**The distribution of Dorsal protein derivatives encoded by introduced transgenes was examined by immunohistochemical staining of syncytial blastoderm embryos using a polyclonal antiserum directed against Dorsal. The production of the antiserum and the protocol used for whole mount stainings is described in Roth et al. (41).

**RESULTS**

**Fusion Proteins Containing the Dorsal prNLS—**The Dorsal-β-galactosidase fusion proteins used in this study are depicted schematically in Fig. 1. The three basic types; full-length Dorsal (D1), the Dorsal rel homology domain (D2), and the Dorsal prNLS (the NLS together with the PKA site, D3), were all able to be phosphorylated by bovine heart PKA C-subunit (data not shown), and to accumulate in the nucleus of HTC rat hepatoma cells in vivo or in vitro (see below). To investigate the role of the PKA site in regulating Dorsal nuclear transport, site-directed mutagenesis was used to substitute Ser312 by either Ala or Glu in all three types of construct, while a mutant NLS form, containing Thr337 and Glu339, was also generated. Negligible phosphorylation of Ala312- or Glu312-substituted mutant derivatives was observed (maximal stoichiometry of phosphorylation of 0.07 ± 0.04 mol Pi/mol tetramer, n = 2, compared with a value of 1.08 ± 0.08 for Ser312 derivatives in the case of D3 constructs), confirming the specificity of PKA phosphorylation at Ser312. The D3 NLS mutant construct was phosphorylated to an extent (1.4 ± 0.03 mol Pi/mol tetramer, n = 2), somewhat

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**Homologous prNLSs in other Dorsal/rel family members:**

| Protein | PK-A site | spacer | NLS |
|---------|-----------|--------|-----|
| c-rel   | RRRS       | -      | -   |
| NF-xB p65 | RRRS       | -      | -   |

**Fig. 1. Sequences of the Dorsal-β-galactosidase fusion protein derivatives used in this study.** All proteins contain β-galactosidase amino acids 9–1023 fused N-terminal to the Dorsal sequences (see “Materials and Methods” for details of plasmid constructions). The sequence of the spacer is identical in all constructs.

**Fig. 2. Nuclear uptake of Dorsal-β-galactosidase fusion protein derivatives in vivo.** A, visualization of nuclear uptake of Dorsal fusion protein derivatives in microinjected HTC cells. Confocal images are shown for cells 20–25 min after microinjection at 37 °C. B, nuclear transport kinetics of Dorsal fusion protein derivatives in microinjected HTC cells as measured by quantitative CLSM. Measurements, performed as described under “Materials and Methods,” represent the average of at least two separate experiments, where each point represents the average of 6–10 separate measurements (S.E. < 9.8% the value of the mean) for each of nuclear (Fn) and cytoplasmic (Fc) fluorescence, respectively, with autofluorescence subtracted. Data were fitted for the function \( F_n(t) = F_{n,\text{max}}(1 - e^{-kt}) \), where \( t \) is time in minutes, \( F_{n,\text{max}} \) is the maximal level of nuclear accumulation, and \( k \) is the first order rate constant (19); collated data are presented in Table I.
higher than that of the wild-type D3 construct, indicating that inactivation of the NLS does not inhibit phosphorylation at Ser312.

Nuclear Import Kinetics of Dorsal Fusion Proteins—The Dorsal-β-galactosidase fusion proteins were assessed for their nuclear import properties at the single-cell level both in vivo and in vitro (Figs. 2 and 3; Table I). The wild-type proteins all accumulated quite strongly, the full length protein being imported to the highest levels with transport half-maximal at about 3 and 16 min in vivo and in vitro respectively (Figs. 2B and 3; Table I). Transport was NLS-dependent, as indicated by results for the NLS mutant D3 construct, where the two point mutations within the NLS completely abolished nuclear accumulation (Figs. 2 and 3). Significantly, transport was also strongly dependent on the PKA site as shown for all (D1–D3) types of construct where substitution of Ser312 by either Ala or Glu essentially abolished nuclear accumulation (Figs. 2 and 3). The Glu312 proteins accumulated to a somewhat higher extent than the Ala312 derivatives, as well as reaching maximal accumulation at a faster rate (Figs. 2B and 3B; Table I), indicating that negative charge at the PKA site to some extent simulates phosphorylation in terms of its enhancing effect on nuclear import.

Nuclear import of the Dorsal fusion proteins in vitro was found to resemble that of T-ag and other NLS-dependent nuclear import pathways in being inhibitable by both the lectin wheat germ agglutinin, which binds to and inactivates nucleoporin components of the nuclear pore complex through which transport takes place (42), and by the nonhydrolyzable GTP analog GTPγS (Table I).

Nuclear Import of Dorsal Fusion Proteins Is Enhanced by cAMP and PKA—To test whether nuclear transport was responsive to cAMP in the case of the wild-type Dorsal proteins, nuclear import kinetics were determined in vitro in the absence and presence of exogenously added PKA C-subunit or cAMP, without and with addition of the PKA-specific inhibitor peptide PKI 5–24 (Fig. 4; Table I; and not shown). In vivo experiments were also performed using cAMP in the microinjection pipette (Table I). In all cases, the nuclear import rate was enhanced by at least 50%, while the maximal level of nuclear accumulation was also slightly increased in the case of the in vitro experiments shown in Fig. 4 (see also Table I). The specificity of this effect was demonstrated by the fact that cAMP did not enhance nuclear import of the NLS-containing fusion protein T-ag-CcN-β-galactosidase, which lacks a PKA site (19).

We have previously shown (19) that quite high endogenous levels of PKA activity are present in intact HTC cells (about 0.5 unit/mg) as well as reticulocyte lysate (the source of cytosol in the in vitro transport assay). This high basal activity is presumably sufficient to effect efficient nuclear import of the fusion proteins in the absence of exogenous hormonal or other stimulation of HTC cells (19). The effects of PKA C-subunit or cAMP addition shown in Fig. 4 and Table I thus almost certainly do not represent a quantitative estimate of the extent of the dependence on PKA activation. Importantly, PKI 5–24 was able to reverse the effects of either PKA C-subunit or cAMP
addition almost completely (Fig. 4 and not shown), indicating that the effects were specific to phosphorylation activity of the PKA C-subunit.

The PKA Site Participates in Recognition of the Dorsal NLS by Importin 58/97—We have recently shown that, in the case of T-ag, phosphorylation at the CK2 site increases the affinity of interaction of the T-ag NLS with the NLS-binding importin (22). To determine whether the PKA site enhances Dorsal nuclear import through an analogous mechanism, we used an ELISA-based assay as described under "Materials and Methods" (22, 34) to determine the affinity of binding of the Dorsal NLS by importin 58 or the PKA C-subunit, and that the PKA site may be directly involved in PKA-mediated enhancement of nuclear transport of Dorsal NLS by importin 58/97. The implication was that negative charge at the PKA site was important in Dorsal nuclear import, it was important to test whether the above observations were physiologically relevant in terms of Dorsal function in Drosophila. The Dorsal nuclear gradient formed during the Drosophila syncytial blastoderm stage is responsible for embryonic dorsal-ventral pattern formation and consequently for the differentiation of dorsal-ventral pattern elements of the first instar larva (44). From ventral to dorsal, the pattern elements defined by the Dorsal gradient are the mesoderm, which gives rise to muscle and a variety of internal organs, the ventral neuroectoderm, which produces the central nervous system and the portion of the ventral hypoderm that includes the conspicuous ventral dens.

In vivo In vitro

| Fusion protein | Nuclear import parameter | Importin 58/97 binding |
|---------------|--------------------------|------------------------|
|               | $K_D$                    | $K_D$ relative wild-type construct |
| D1 (wild type) | 3.35 ± 0.15              | 26.3 ± 8.9 (3) $^d$    |
| D1 (Ala312)   | ND                       | 142 ± 11.5 (2)         |
| D1 (Glu312)   | ND                       | 67.0 ± 22.0 (2)        |
| D2 (wild type) | 2.12 ± 0.09              | 35.9 ± 13.0 (3) $^f$  |
| D2 (Ala312)   | 0.59 ± 0.01              | 191 ± 641 (3)         |
| D2 (Glu312)   | 0.65 ± 0.04              | 54.6 ± 11.5 (3)        |
| D3 (wild type) | 2.02 ± 0.08              | 48.5 ± 5.2 (4) $^d$  |

$^a$ The Dorsal β-galactosidase fusion protein sequences are shown in Fig. 1. The T-ag CcN-β-galactosidase fusion protein contains T-ag amino acids 111–135, including the CcN motif and NLS (13).

$^b$ Raw data (see Figs. 2B and 3B, and not shown) were fitted as described in the legend to Fig. 2B (19). Results shown are averaged over at least two separate experiments with the S.E. indicated.

$^c$ Binding of Dorsal β-galactosidase fusion proteins to mouse importin 58 and 97 subunits and the 58/97 complex was quantitated using an ELISA-based binding assay as described under "Materials and Methods" (22, 34). Curves (see Fig. 5 and not shown) were fitted for the function $B(s) = B_{max} (1 - e^{-ks})$, where $x$ is the concentration of importin subunits/complex. Results represent the mean ± S.E. for at least two separate experiments, where the number of experiments is shown in parentheses. Maximal importin binding by all Dorsal proteins was similar, with the exception of the D3 (NLS mutant) protein whose maximal binding was 2.4 ± 1.2% that of D3 (wild type).

$^d$ Denaturation at 65 °C resulted in an over 20-fold increase in $K_D$.

$^e$ ND, not determined.

$^f$ Apyrase pretreatment was used and the ATP-regenerating system omitted (see "Materials and Methods").

$^g$ WGA, wheat germ agglutinin.

Consistent with the results for nuclear import kinetics (above), the Ala312 PKA site variants exhibited greatly reduced binding affinities (4–5-fold higher $K_D$ values) compared with the wild-type derivatives (Fig. 5; Table I). The Glu312 variants exhibited 2–3-fold higher affinity for importin than the Ala312 variants. The implication was that negative charge at the PKA site enhances recognition of the Dorsal NLS by the importin complex, and that the PKA site may be directly involved in NLS binding by importin. It was concluded that the basis of PKA-mediated enhancement of nuclear transport of Dorsal fusion proteins is modulation of the affinity of binding of the Dorsal NLS by importin 58/97.
which normally produce embryos that are incapable of defining a polarized dorsal-ventral axis because of the lack of Dorsal (see Fig. 7B), and differentiate as apolar tubes of cuticle of the type found on the dorsal side of wild-type embryos (Fig. 6B). This is in contrast to \( dl^{1}/dl^{1} \) mutant females that carry a wild-type dorsal transgene, which gives rise to hatching larvae with all of the dorsal-ventral pattern elements seen in wild-type larvae (Fig. 6A). These were compared with the progeny of females expressing dorsal transgenes (see “Materials and Methods”) containing mutations either in the NLS (Thr\(^{337}\) Glu\(^{339}\)) or the PKA site Ser\(^{312}\) identical to those within the Dorsal fusion protein derivatives examined above. All of the progeny of the \( dl^{1}/dl^{1} \) females expressing mutant dorsal transgenes were found to be incapable of defining a complete dorsal ventral axis, embryos from females expressing the NLS-deficient mutation exhibiting the most severe defects. Although they produced tracheal filzkörper material (Fig. 6C), these embryos never produced ventrolaterally or ventrally derived pattern elements such as ventral denticles or muscle, consistent with our inability to detect nuclear localization of the mutant Dorsal protein in the embryos (Fig. 7D).

Embryos derived from the most strongly rescuing transgenic lines carrying the PKA mutations (Ala\(^{312}\) or Glu\(^{312}\)) did develop ventrolateral and dorsolateral pattern elements in the absence of the wild-type Dorsal protein. The most strongly rescued embryos from the PKA site mutant expressing females produced ventral denticle material as well as filzkörper (Fig. 6, D and E) but did not hatch and did not exhibit movement within their egg shells, suggesting that they were unable to form mesoderm, the most ventrally derived pattern element. The strong correlation between the impairment, effected by the PKA site and NLS mutations within the Dorsal prNLS, of nuclear localization as shown in rat hepatoma cells (above), and of \textit{Drosophila} development as indicated by these phenotypes, implies physiological relevance of the former results.

Decreased nuclear localization of the Dorsal protein in the case of which normally produce embryos that are incapable of defining a polarized dorsal-ventral axis because of the lack of Dorsal (see Fig. 7B), and differentiate as apolar tubes of cuticle of the type found on the dorsal side of wild-type embryos (Fig. 6B). This is in contrast to \( dl^{1}/dl^{1} \) mutant females that carry a wild-type dorsal transgene, which gives rise to hatching larvae with all of the dorsal-ventral pattern elements seen in wild-type larvae (Fig. 6A). These were compared with the progeny of females expressing dorsal transgenes (see “Materials and Methods”) containing mutations either in the NLS (Thr\(^{337}\) Glu\(^{339}\)) or the PKA site Ser\(^{312}\) identical to those within the Dorsal fusion protein derivatives examined above. All of the progeny of the \( dl^{1}/dl^{1} \) females expressing mutant dorsal transgenes were found to be incapable of defining a complete dorsal ventral axis, embryos from females expressing the NLS-deficient mutation exhibiting the most severe defects. Although they produced tracheal filzkörper material (Fig. 6C), these embryos never produced ventrolaterally or ventrally derived pattern elements such as ventral denticles or muscle, consistent with our inability to detect nuclear localization of the mutant Dorsal protein in the embryos (Fig. 7D).

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are oriented as in Fig. 6. Maternal genotypes: stainings were carried out using an antibody directed against the Dor-

Glu312). The p(wbcd

Similar to the predominantly cytoplasmic NLS mutant (Fig. 7), while the arrowheads indicate the position of ventral denticle bands.

The arrows indicate the position of tracheal filzkörper material (out of the plane of focus in E), while the arrowheads indicate the position of ventral denticle bands.

PKA Site Enhancement of Dorsal NLS Recognition

This study represents the first determination of the nuclear import kinetics of the Drosophila TF Dorsal, defining its prNLS as being functional in nuclear targeting in cAMP-responsive fashion in higher mammals. Dorsal amino acids 297–346 are clearly sufficient to confer NLS- and PKA-site-dependent nuclear localization on the heterologous protein β-galactosidase, as well as to mediate binding to importin. Our experiments show that nuclear import of Dorsal fusion proteins is inhibi-

In considering the results here for the Dorsal prNLS, it should be remembered that the experiments were performed in rat hepatoma cells which lack specific components, such as Cactus and Pelle, which are involved in the regulation of Dorsal subcellular localization during Drosophila embryonic development. There does, however, appear to be some evidence for promiscuity in rel TF and inhibitor family member binding (e.g.
Cactus appears to be able to bind and functionally interact with NF-κB p65-27. It seems reasonable to hypothesize that the mechanistic basis of regulation of nuclear import by the Dorsal prNLS is essentially independent of these factors. Indeed, the results of this study clearly suggest that the Dorsal PKA site regulates NLS recognition by the importin subunits. While the Toll signaling pathway revolves around phosphorylation-induced release of Dorsal from Cactus through the action of Pelle (28, 29, 31, 32), there is clear evidence from in vitro studies of phosphorylation of Dorsal subsequent to dissociation from Cactus (29, 45, 46). It thus seems reasonable to postulate that in analogous fashion to the NF-κB proteins (2, 3, 10), at least two pathways involving phosphorylation, one of them mediated by the prNLS characterized here, control Dorsal nuclear localization (45). This is also consistent with genetic evidence that suggests that degradation of Cactus is necessary but not sufficient to induce complete Dorsal nuclear localization (45).

The question of whether PKA is actually the kinase that phosphorylates Ser312 in vivo and thereby regulates Dorsal nuclear import is complicated in part by the fact that there are two known forms in Drosophila (47, 48). Although PKA-DC2 deficiency appears to have little effect on viability (47), flies homozygous for null alleles of PKA-DCO die as first instar larvae (48), thus preventing examination of the progeny of adult females. Females heterozygous for weak DC0 alleles produce offspring showing a variety of defects in embryogenesis (48), including preblastoderm arrest and alterations in cuticular patterning. In short, there appears to be no definitive genetic evidence either for or against the idea that PKA is the kinase responsible for the effects on Dorsal nuclear import in vivo, leaving open the possibility that other kinase(s) able to phosphorylate Ser312 could well play the key physiological role.

Importantly, this study indicates that a Drosophila NLS and fascinatingly its regulatory mechanisms are functional in mammalian cells. These results are similar to those for SWI5 (see Introduction), supporting the idea that phosphorylation and prNLSs are mechanisms for regulating nuclear protein import that are conserved across eukaryotes from yeast to flies to higher mammals (2, 3, 10). Regulation of the activities of the kinases that specifically phosphorylate at a particular prNLS through hormones, growth factors, and so forth in turn determines the nuclear import of the proteins carrying these prNLSs (2, 3), ultimately thus constituting one of the central mechanisms by which eukaryotic cell gene expression can be modulated.

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