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Transport of DNA into the Nuclei of Xenopus Oocytes by a Modified VirE2 Protein of Agrobacterium

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We used Agrobacterium T-DNA nuclear transport to examine the specificity of nuclear targeting between plants and animals and the nuclear import of DNA by a specialized transport protein. Two karyophilic Agrobacterium virulence (Vir) proteins, VirD2 and VirE2, which presumably associate with the transported T-DNA and function in many plant species, were microinjected into Drosophila embryos and Xenopus oocytes. In both animal systems, VirD2 localized to the cell nuclei and VirE2 remained exclusively cytoplasmic, suggesting that VirE2 nuclear localization signals may be plant specific. Repositioning one amino acid residue within VirE2 nuclear localization signals enabled them to function in animal cells. The modified VirE2 protein bound DNA and actively transported it into the nuclei of Xenopus oocytes. These observations suggest a functional difference in nuclear import between animals and plants and show that DNA can be transported into the cell nucleus via a protein-specific pathway.

INTRODUCTION

Agrobacterium is a phytopathogen that elicits neoplastic growths on many plant species. This genetic transformation of plants is achieved by transferring a single-stranded copy of the bacterial T-DNA (the T-strand) from the Ti plasmid into the plant cell nucleus (Tinland et al., 1994; Yusibov et al., 1994; Citovsky and Zambryski, 1995). Nuclear import of the T-strand is most likely mediated by two Agrobacterium virulence (Vir) proteins, VirD2 and VirE2 (reviewed in Citovsky and Zambryski, 1993, 1995). Presumably, both proteins associate directly with the transported T-strand to form the T-complex (Howard and Citovsky, 1990; Howard et al., 1990). In this complex, one molecule of VirD2 is attached covalently to the 5′ end of the T-strand (Herrera-Estrella et al., 1988; Ward and Barnes, 1988; Young and Nester, 1988; Howard et al., 1989), whereas VirE2, a single-stranded DNA (ssDNA) binding protein (SSB) (Gietl et al., 1987; Christie et al., 1988; Citovsky et al., 1988, 1989), is thought to coat the rest of the ssDNA molecule cooperatively (Citovsky et al., 1989).

Recent studies have demonstrated that VirD2 and VirE2 are karyophilic, presumably acting to transport the associated T-strand into the plant cell nucleus (Herrera-Estrella et al., 1988; Citovsky et al., 1992b, 1994; Howard et al., 1992). The nuclear localization signal (NLS) sequences of VirD2 and VirE2 have been identified and characterized in detail; specifically, VirD2 has been shown to contain one bipartite NLS (Howard et al., 1992), and VirE2 has been shown to have two independently active bipartite NLSs (Citovsky et al., 1992b). VirD2 and VirE2 NLSs function in various plant species; furthermore, the NLSs of both proteins are active even in plants recalcitrant to Agrobacterium infection (Citovsky et al., 1994), indicating the universal character of these signals in plants.

The T-DNA element itself presumably does not carry signals for nuclear transport because it is sequence nonspecific; any DNA placed between the T-DNA borders of the Ti plasmid will be transported into the plant cell nucleus and function as the T-DNA (reviewed in Zambryski, 1992). Thus, the VirD2 and VirE2 NLSs are most likely the only signals required for T-DNA nuclear import. This role of VirD2 and VirE2 in T-DNA nuclear uptake, however, was inferred from their proposed association with the transported DNA (Citovsky et al., 1992b; Howard et al., 1992) and has not been demonstrated directly. In fact, the hypothesis that a specific protein can physically transport DNA into the cell nucleus has not been proven. A few studies implicating proteins in DNA nuclear import used either nonspecific nuclear proteins (Kaneda et al., 1989) or the entire particles of the simian virus 40 (Clever et al., 1991). In contrast, T-DNA nuclear import is a simple and well-defined experimental system to study protein-mediated transport of DNA molecules across the nuclear envelope.

Here, we used this system to determine whether plant NLSs are active in animal cells and to assay directly protein-mediated transport of DNA into the cell nucleus. Specifically, we examined the function of NLSs of Agrobacterium VirD2 and VirE2 proteins in Drosophila embryos and Xenopus oocytes. We also tested whether the VirE2 protein can carry DNA into the nuclei of Xenopus oocytes. Together with our previous observations (Citovsky et al., 1992b, 1994; Howard et al., 1992), our results indicate that the VirD2 NLS functions in both plant and animal cells, whereas the NLSs of VirE2 are active in plant but

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not in animal systems. Repositioning of a single amino acid residue within the VirE2 NLS allowed this signal to function in both Drosophila and Xenopus cells. This modified VirE2 was then used to deliver DNA into the nuclei of Xenopus oocytes via a protein NLS-specific pathway.

RESULTS

Nuclear Import of VirD2 in Drosophila Embryos

We used VirD2 and VirE2 to determine whether their NLSs are able to function in evolutionarily distant animal cells. To this end, VirD2 and VirE2 were produced in *Escherichia coli* and purified to near homogeneity. The purified proteins were tagged fluorescently at cysteine residues; the cysteine-specific labeling avoids modification of basic residues critical for NLS activity. The fluorescent proteins were then microinjected into 1.5- to 2-hr-old Drosophila embryos. At this developmental stage, Drosophila embryos contain 750 to 6000 nuclei in a syncytium (no individual cell membranes); most of these nuclei are at the embryo surface and easily visualized (reviewed in Campos-Ortega and Hartenstein, 1985). Figures 1A and 1B show that after microinjection, rhodamine-labeled VirD2 accumulated in Drosophila nuclei. This nuclear import was very efficient because virtually all observed nuclei took up the label (Figures 1A and 1B). The nuclear location of the fluorescent protein was confirmed by costaining with the DNA binding dye oligreen (Molecular Probes, Inc., Eugene, OR; data not shown).

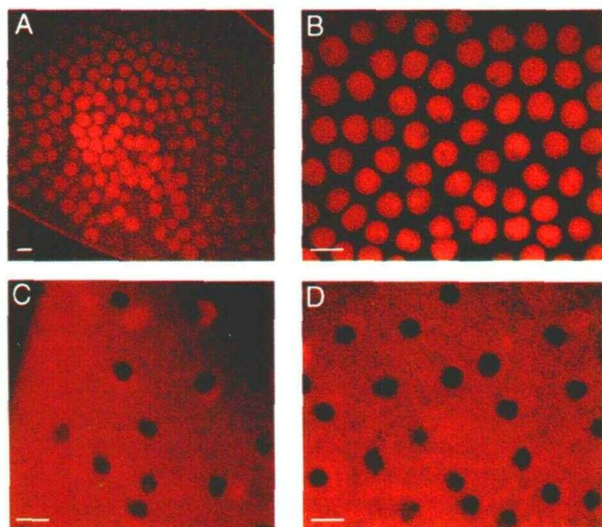

Figure 1. Nuclear Import of VirD2 in Drosophila Embryos.

(A) and **(B)** Rhodamine-labeled VirD2 microinjected into Drosophila embryos alone.

(C) Rhodamine-labeled VirD2 microinjected into Drosophila embryos in the presence of 1 mM GTPγS.

(D) Rhodamine-labeled VirD2 microinjected into Drosophila embryos in the presence of competing amounts of a synthetic VirD2 NLS peptide. Bars = 10 μm.

Next, we tested whether the nuclear accumulation of the label reflected an active process of nuclear import. Because such a process always requires NLS (reviewed in Garcia-Bustos et al., 1991; Forbes, 1992), it should be specifically inhibited by competing amounts of the free signal peptide, which presumably saturates the nuclear import machinery (Michaud and Goldfarb, 1991; Guilizia et al., 1994). We coinjected VirD2 with a 30-fold molar excess of a synthetic peptide, corresponding to the VirD2 NLS. As shown in Figure 1D, VirD2 nuclear import was blocked completely by this peptide. Furthermore, VirD2 nuclear uptake also was inhibited by coinjection of a nonhydrolyzable analog of GTP (GTPγS) (Figure 1C). GTPγS has been shown to block the Ran/TC4 GTPase, which is absolutely essential for the transport of proteins through the nuclear pore complex (Melchior, 1993; Moore and Blobel, 1993; Goldfarb, 1994). Collectively, these results indicate that VirD2 is actively imported into the nuclei of Drosophila embryos and that this import is mediated specifically by the VirD2 NLS.

To characterize further the dynamics of VirD2 nuclear import in Drosophila, the microinjected embryos were recorded using time lapse video microscopy. The VirD2 fluorescence intensity within each of 20 randomly chosen nuclei was monitored from the moment of injection. The kinetics of VirD2 nuclear import were determined by comparing the amount of fluorescence in the cytoplasm with that in the individual nuclei at different time periods. These experiments showed that practically all of the injected VirD2 accumulated in the Drosophila nuclei within 10 min after microinjection (Figure 2). This time course of the VirD2 nuclear import is comparable with that reported for animal NLS-containing proteins (Rihs and Peters, 1989; Rihs et al., 1991). Thus, the results in Figures 1 and 2 establish that the plant NLS residing in the VirD2 protein is active in an animal system.

VirE2 Remains Cytoplasmic in Drosophila Embryos

VirD2 is a minor component of the T-complex compared with VirE2. For example, a nopaline-type Agrobacterium is thought to produce a T-complex containing only one molecule of VirD2 and ∼600 molecules of VirE2 (reviewed in Citovsky and Zambryski, 1993). Deletion of the VirD2 NLS only partially inhibits Agrobacterium tumorigenicity (Shurvinton et al., 1992), suggesting an important role for VirE2 in this process; consistent with these observations, VirE2 accumulates efficiently in the cell nuclei in various plant species (Citovsky et al., 1992b, 1994). To test whether this protein also functions in animal cells, we used fluorescently labeled VirE2 to assay the activity of its NLSs in Drosophila embryos. To our surprise, VirE2 remained cytoplasmic in Drosophila (Figure 3A). This complete absence of nuclear import demonstrates that the VirE2 NLSs are not recognized in this animal system.

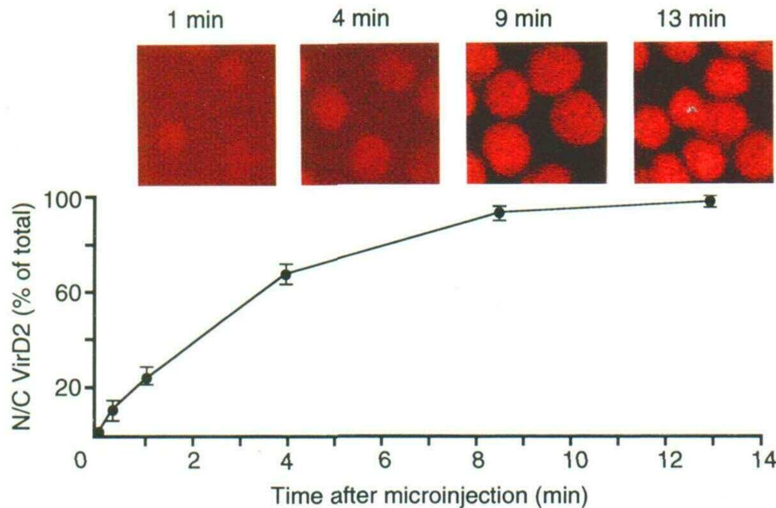

Figure 2. Kinetics of VirD2 Nuclear Import.

Drosophila embryos were microinjected with the fluorescently labeled VirD2, and the intracellular fluorescence was recorded by using time lapse video. The intensity of intranuclear and cytoplasmic fluorescence was quantified using images recorded at the indicated time points, and the degree of VirD2 nuclear import was expressed as a ratio of the nuclear and cytoplasmic fluorescence. Each point of the curve represents the average of 20 different nuclei. N/C indicates the ratio of the intensities of the fluorescent signal in the cell nucleus and the cell cytoplasm.

Single Amino Acid Changes in NLSs of VirE2 Promote Its Nuclear Import in Drosophila Embryos

To address the question of what is the molecular basis for the functional difference between the VirD2 and VirE2 NLSs, we compared the amino acid sequence of both VirE2 NLSs with the VirD2 NLS and with the NLS of Xenopus nucleoplasmin (Robbins et al., 1991), a paradigm for the bipartite NLS sequence found in the large majority of nuclear-targeted proteins (Dingwall and Laskey, 1991). Figure 4 shows that although VirD2 and VirE2 NLSs are generally homologous to the bipartite NLS of nucleoplasmin, the similarity of the VirD2 NLS to nucleoplasmin is greater than that of the VirE2 NLSs. Specifically, bipartite NLSs are composed of two basic domains separated by a variable-length spacer (usually nine to 11 residues). The first domain contains two adjacent basic amino acid residues, and the second domain contains five residues, three of which are basic (Dingwall and Laskey, 1991; Robbins et al., 1991). As shown in Figure 4, the VirD2 NLS fits the bipartite motif perfectly. In contrast, the VirE2 NLSs (previously designated NSE1 and NSE2; Citovsky et al., 1992b) have a consensus-type second domain but differ in their first domains. Both NSE1 and NSE2 have an uncharged amino acid residue positioned between the two basic residues of the first domain (Figure 4). It is possible that this single amino acid deviation from the consensus motif underlies the inability of the VirE2 NLSs to function in Drosophila, rendering it unrecognizable by the Drosophila nuclear import machinery.

To test this idea, we produced VirE2 mutants in which the intervening uncharged amino acid residue was switched with the adjacent basic residue of the first domain. Specifically, the leucine residue of NSE1 was switched with the adjacent arginine, changing the first domain sequence from KLR to KRL (mutant VirE2s11; Figure 4), and the threonine residue of NSE2 was switched with the adjacent lysine, changing the first domain from KTK to KKT (mutant VirE2s20; Figure 4). The VirE2s11 and VirE2s20 mutant proteins were then labeled fluorescently and microinjected into Drosophila embryos. The results in Figures 3B and 3C show that both VirE2s11 and VirE2s20 localized to the Drosophila nuclei.

We also observed that although the imported VirD2 was distributed randomly within the Drosophila nuclei (Figure 1B), the s11 and s20 mutant versions of VirE2 accumulated in distinct subdomains inside the nuclei (Figures 3B and 3C). Because VirE2 is a sequence-nonspecific SSB (Gietl et al., 1987; Christie et al., 1988; Citovsky et al., 1988, 1989) and most SSBs also bind RNA and double-stranded DNA with low affinity (reviewed in Chase and Williams, 1986), it is possible that this staining pattern may represent the association of VirE2 with the nuclear DNA and/or RNA. In contrast, VirD2 should not bind nucleic acids because its association with DNA is limited to a specific 25-bp sequence found in the T-DNA borders (reviewed in Zambryski et al., 1989; Citovsky et al., 1992a; Zambryski, 1992).

It is important to note that all of our microscopic data are confocal optical sections with the plane of focus through the

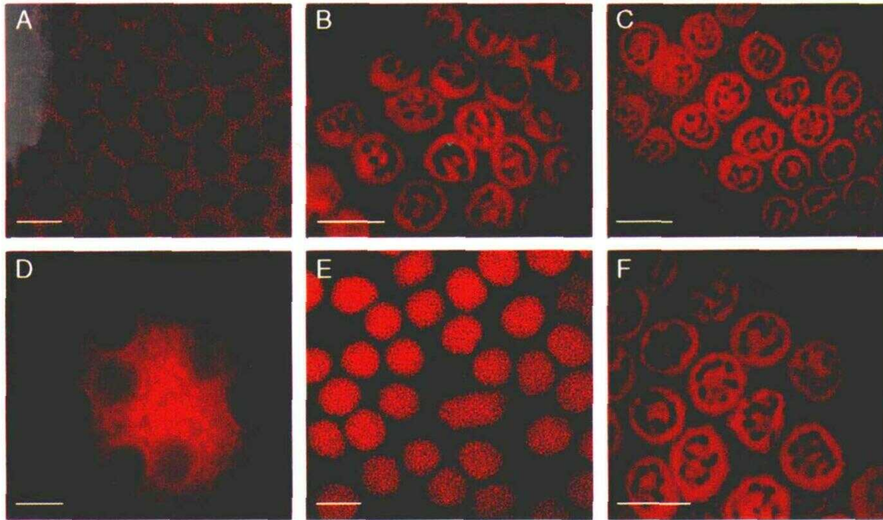

Figure 3. Nuclear Import of VirE2, VirE2s11, and VirE2s20 in Drosophila Embryos.

(A) Wild-type VirE2.
(B) VirE2s11.
(C) and **(D)** VirE2s20.
(E) VirD2.
(F) VirE2s20.
VirE2, VirE2s11, and VirE2s20 were fluorescently labeled and microinjected into Drosophila embryos either alone (**[A]** to **[C]**) or in the presence of competing amounts of the synthetic VirD2 NLS peptide **(D)** or the VirE2 NSE2 peptide (**[E]** and **[F]**). Bars = 10 μm.

	first domain		second domain
wt VirE2 NSE1	KlR	pedryiqte-	KygRR
s11	KR	l·········-	KygRR
wt VirE2 NSE2	KtK	ygsdtei---	KlKsK
s20	KK	t·······---	KlKsK
VirD2	KR	predddgepse	RKReR
nucleoplasmin	KR	paatkkagqa-	KKKKl

Figure 4. Amino Acid Sequence Comparison of the NLSs of VirE2, VirE2s11, VirE2s20, and VirD2 with the Consensus Bipartite NLS of Nucleoplasmin.

The amino acid sequences of VirE2 and VirD2 bipartite NLSs were determined previously (Citovsky et al., 1992b; Howard et al., 1992, respectively), and the nucleoplasmin NLS was described by Dingwall and Laskey (1991) and Robbins et al. (1991). Boxed residues indicate the first and the second domains of the bipartite NLS. Amino acid residues are shown in one-letter code. Uppercase letters indicate basic amino acid residues of the first and second domains, and lowercase letters indicate all other amino acid residues. Dashes indicate alignment gaps in the amino acid composition of the spacer region between the first and the second domains. Dots indicate identical amino acid residues.

cell nuclei. Thus, the fluorescent staining of the Drosophila nuclei after microinjection of VirD2, VirE2s11, and VirE2s20 most likely reflects protein accumulation within the nuclei rather than simply perinuclear binding. However, the microinjected VirE2s20 and VirE2s11 displayed a ringlike staining in addition to the central staining of the nucleus (Figures 3B and 3C). It is possible, therefore, that only a portion of these mutant proteins was translocated into the nucleus, whereas some of the fluorescent label remained bound to the outer surface of the nuclear envelope.

Probing the Specificity of Nuclear Import by Competition with Synthetic NLS Peptides

Synthetic NLS peptides coinjected with a nuclear-targeted protein inhibit its nuclear transport, presumably by competing for the NLS-binding receptor (Michaud and Goldfarb, 1991; Guilizia et al., 1994). Thus, these peptides can be used to assay for the presence of cellular receptors recognizing specific NLS sequences; only peptides that compete for the same receptor as the tested NLS will inhibit its import into the nucleus.

We used this approach to support the idea that VirD2 but not VirE2 NLSs function in Drosophila. A synthetic peptide cor-

responding to the second VirE2 NLS (NSE2; Citovsky et al., 1992b) was coinjected with the rhodamine-labeled VirD2. This peptide failed to inhibit nuclear import of VirD2 (Figure 3E). The coinjected NSE2 peptide also did not affect nuclear accumulation of VirE2s20 (Figure 3F). These results indicate that the VirE2 NLS is not recognized by the Drosophila nuclear transport machinery. In contrast, the VirD2 NLS peptide efficiently inhibited nuclear transport of both VirD2 (Figure 1C) and VirE2s20 (Figure 3D).

Nuclear Import of VirD2 and VirE2 in Xenopus Oocytes

Our results indicate that the NLS signals of VirE2 do not function in animal cells and thus may be plant specific. To determine the generality of this observation, we tested the nuclear transport of VirE2 and VirE2s20 in an unrelated animal system, Xenopus oocytes. Figure 5A shows that VirD2 was transported efficiently into the cell nucleus after microinjection into a Xenopus oocyte. Similarly, efficient nuclear import was observed with the VirE2s20 protein (Figure 5C), whereas the wild-type VirE2 was excluded from the nucleus and remained cytoplasmic (Figure 5B). The position of the nucleus in these experiments was confirmed by using the chromatin-specific stain 4',6-diamidino-2-phenylindole (DAPI) (data not shown).

VirE2s20 Protein Delivers DNA into the Nuclei of Xenopus Oocytes

Because VirE2 is both a nuclear-targeted protein and an SSB, it may function to transport ssDNA into the cell nucleus. Here, we tested this hypothesis directly. First, we assayed the ssDNA binding of the wild-type VirE2 protein and its s11 and s20 derivatives. Figure 6C shows that the SSB activity of VirE2s11 was lower than that of the wild-type VirE2 (Figure 6B), confirming earlier observations that VirE2 NLSs partially overlap their ssDNA binding site (Citovsky et al., 1992b, 1994). In contrast, the SSB activity of VirE2s20 was indistinguishable from that of the wild-type protein (Figures 6D and 6B, respectively). Both VirE2 and VirE2s20 saturated the ssDNA probe at an ~10:1 (w/w) ratio, consistent with previous characterization of the wild-type VirE2 protein (Citovsky et al., 1989). These results suggest that the s11 mutation may have caused some conformational changes in VirE2, although the s20 mutation most likely did not interfere with the protein conformation.

Based on these observations, we used VirE2s20 to test its ability to transport ssDNA into the cell nucleus. To this end, ssDNA was mixed in vitro with the purified VirE2s20 to form VirE2s20–ssDNA complexes. Because such complexes are large (~2.5×10^6 D per 1 kb of DNA; Citovsky and Zambryski, 1993), they frequently clog microinjection needles (B. Guralnick and V. Citovsky, unpublished observations). To circumvent this technical difficulty, we chose to microinject VirE2s20–ssDNA complexes into Xenopus oocytes; microinjection into these cells allows the use of relatively wide needle bores. The nuclear import of VirE2s20–ssDNA complexes was assayed directly using fluorescently labeled DNA. The results in Figure 7A show that the fluorescent ssDNA alone does not enter the cell nucleus after microinjection into the oocyte cytoplasm. Under the same conditions, VirE2s20–ssDNA complexes were imported efficiently into the oocyte nucleus (Figure 7C). Nuclear accumulation of the fluorescent ssDNA complexed with VirE2s20 was similar to that achieved by microinjecting the ssDNA alone into the oocyte nucleus (compare Figures 7B and 7C). The position of the nucleus in oocyte cells was confirmed by cytoplasmic microinjections of the chromatin-specific stain DAPI (see Figure 7H for one such DAPI-stained oocyte nucleus).

VirE2s20-mediated nuclear import of ssDNA was confirmed further by using the nuclei isolated from the microinjected oocytes. When the nuclei from the oocytes injected with the fluorescent VirE2s20–ssDNA complexes were removed and examined directly under a confocal microscope, they contained the fluorescent signal (Figure 7E). In contrast, the nuclei from cells microinjected with the fluorescent ssDNA alone displayed no fluorescence above the background level (Figure 7F). The nuclear import of VirE2s20–ssDNA complexes was inhibited

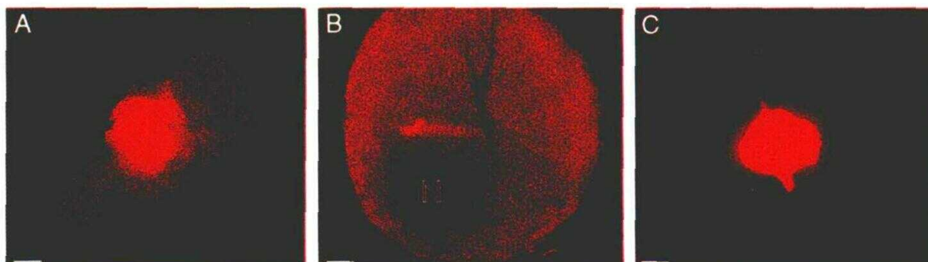

Figure 5. Nuclear Import of VirD2, VirE2, and VirE2s20 in Xenopus Oocytes.

(A) VirD2.

(B) VirE2. N indicates the position of the unstained oocyte nucleus, as determined by the chromatin-specific DAPI staining.

(C) VirE2s20.

All injections were cytoplasmic. Bars = 100 μm.

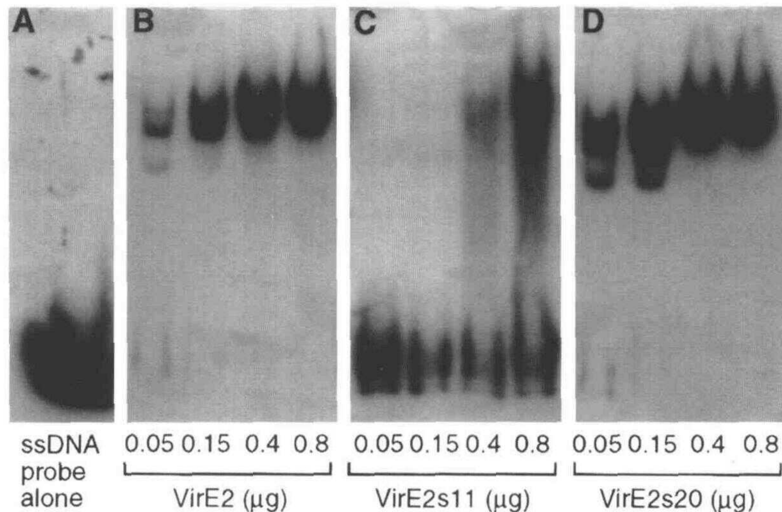

Figure 6. ssDNA Binding Activity of VirE2 and Its Derivatives.

(A) No protein.
(B) VirE2.
(C) VirE2s11.
(D) VirE2s20.
For experimental details, see Methods.

by coinjection of the synthetic VirD2 NLS peptide (Figure 7D). These results demonstrate that the VirE2s20 mutant protein is able to transport ssDNA into the cell nucleus and that this transport most likely occurs by a protein NLS-dependent pathway.

Finally, we tested whether physical association between an NLS-containing protein and DNA is required for DNA nuclear import. Agrobacterium VirD2 was used as an example of nuclear-targeted protein that does not bind DNA. Similar to VirE2s20, VirD2 has a functional consensus bipartite NLS (Figure 4) and localizes efficiently to the animal cell nuclei (see Figures 1A, 1B, and 5A). Unlike VirE2s20, however, VirD2 binding to DNA is highly specific and is limited to the double-stranded 25-bp sequence of the T-DNA borders (reviewed in Zambryski et al., 1989; Citovsky et al., 1992a; Zambryski, 1992). Thus, VirD2 is not expected to form complexes with the fluorescently labeled ssDNA probe that does not contain these borders. When this fluorescent ssDNA was mixed with VirD2 and microinjected into the oocyte cytoplasm, the DNA remained exclusively cytoplasmic (Figure 7G). This observation suggests that DNA molecules must be complexed with the transport protein (such as VirE2s20) to enter the nucleus.

DISCUSSION

VirD2 and VirE2 NLSs Represent Two Functional Types of Plant Nuclear Targeting Signals

Although the function and structure of protein NLSs have been studied extensively in practically all eukaryotic systems, the possibility of NLS specificity between animals and plants had not been examined in detail. Another long-standing question is whether a specific NLS-containing protein can mediate nuclear import of DNA molecules directly. Here, we used the nuclear import of Agrobacterium VirD2 and VirE2 as an experimental system to address these questions. Our results provide evidence for two functional types of plant NLSs: (1) a general type, exemplified by the VirD2 NLS, which is active both in plant and animal systems; and (2) a potentially plant-specific NLS, such as the NSE1 and NSE2 signals of VirE2, that is active in many plant species but nonfunctional in animal systems. Structurally, the VirD2 general NLS conforms precisely to the bipartite NLS sequence as defined for Xenopus nucleoplasmin (Dingwall and Laskey, 1991; Robbins et al., 1991); in contrast, the VirE2 NLS deviates from the bipartite NLS in its first domain. Interestingly, both VirE2 NLSs differ from the VirD2 NLS and from the bipartite consensus in the position of only one amino acid. Our results indicate that one uncharged amino acid residue positioned between two basic residues of the first bipartite domain does not interfere with the NLS function in plant cells (Citovsky et al., 1992b, 1994) but completely blocks the NLS activity in animal systems. Repositioning this intervening amino acid outside the first bipartite domain converts the VirE2 NLS into the general type, which is active in both plant and animal systems.

The wild-type VirE2 protein has two independently active NLSs (Citovsky et al., 1992b). Multivalent NLSs often are more active than the signal monomers (Gerace and Burke, 1988). However, because both VirE2 NLSs together do not function in animal cells, these signals may be genuinely different from their animal counterparts. Additional support for this idea is derived from the observations that the yeast Matα2 NLS func-

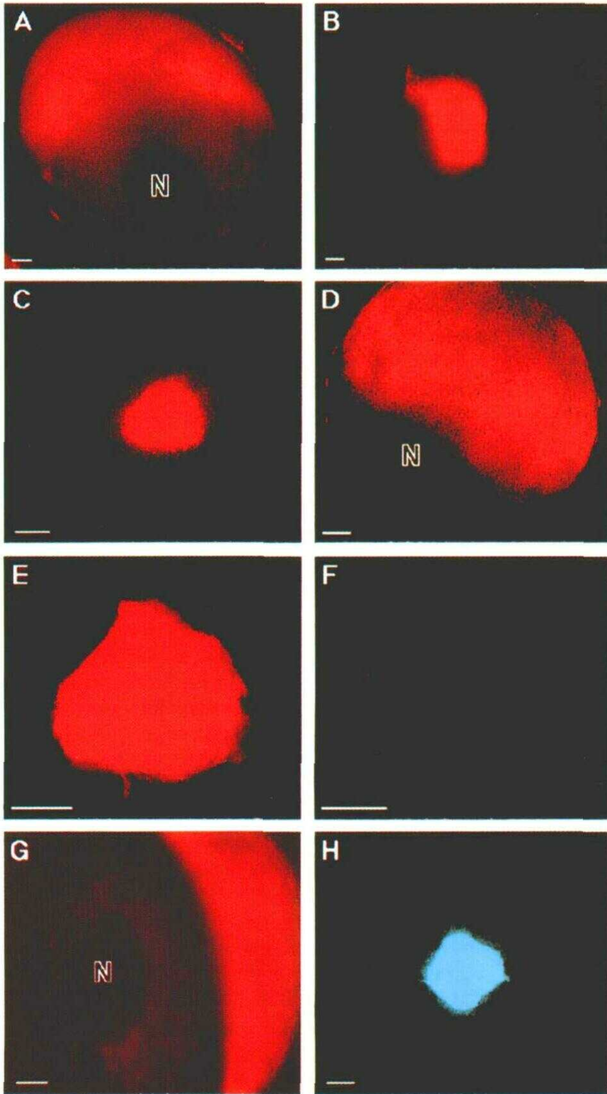

Figure 7. Nuclear Import of VirE2s20–ssDNA Complexes in Xenopus Oocytes.

(A) and **(B)** Free fluorescently labeled ssDNA complexes microinjected into the cytoplasm or into the nucleus of Xenopus oocytes, respectively.
(C) VirE2s20–ssDNA complexes microinjected into the oocyte cytoplasm.
(D) VirE2s20–ssDNA complexes mixed with the synthetic VirD2 NLS peptide microinjected into the oocyte cytoplasm.
(E) and **(F)** Nuclei isolated from oocytes microinjected with VirE2s20–ssDNA complexes or with ssDNA alone, respectively.
(G) VirD2 mixed with the fluorescent ssDNA and microinjected into the oocyte cytoplasm.
(H) DAPI stain image of an oocyte cell.
N indicates the position of the unstained oocyte nucleus, as determined by the chromatin-specific DAPI staining. Bars = 100 μm.

tions in plants (Hicks et al., 1995) but not in mammalian cells (Chelsky et al., 1989; Lanford et al., 1990), indicating differences between plant and animal nuclear import machinery.

Implications for the T-DNA Nuclear Import

The mechanism by which cells distinguish between the VirD2 and VirE2 NLSs is not known. This recognition most likely occurs at the level of interaction between the NLS and its cellular receptors (reviewed in Nigg et al., 1991; Dingwall and Laskey, 1992; Yamasaki and Lanford, 1992; Powers and Forbes, 1994). Thus, it is possible that plant cells have a subset of NLS receptors, potentially belonging to the karyopherin α/importin 60 or Kap60 (former yeast Srp1) protein families (Powers and Forbes, 1994; Enenkel et al., 1995; Rexach and Blobel, 1995), which recognize the VirE2 NLSs and are absent in animal cells. Other plant cell NLS receptors may recognize the general bipartite-type NLS of VirD2, sharing this recognition with the animal NLS binding proteins. Alternatively, the same plant NLS receptors may recognize both the VirD2 and VirE2 NLSs but with different affinity, whereas animal receptors are more stringent, interacting only with the consensus NLS sequences. In both scenarios, functional variations in the NLS sequence may reflect cellular regulation of nuclear import of both proteins and protein–nucleic acid complexes. For example, the nuclear import of Agrobacterium T-complex has been proposed to occur in a polar and linear fashion (reviewed in Zambryski, 1992; Citovsky and Zambryski, 1993, 1995). This polarity of nuclear import may be important for the subsequent integration of the T-strand into the plant cell genome. Indeed, the genetic studies agree that the T-strand integration is a polar process; however, it is still not clear whether it begins at the 5′ or the 3′ end of the T-strand (Citovsky and Zambryski, 1995; Tinland and Hohn, 1995). In any case, there must be a mechanism to differentiate between the 5′ and the 3′ ends of the imported T-strand molecule.

Our model of the T-complex (Howard et al., 1990; Citovsky and Zambryski, 1993, 1995) suggests that the 5′ end of the T-strand is associated with the VirD2 molecule, whereas the 3′ end most likely has a VirE2 molecule attached in its proximity; the functional variation between the NLS signals of these proteins may specify the ends of the T-strand and determine the polarity of its transport and integration. Polar translocation may be a common feature of transport through the nuclear pore complex for naturally occurring nucleic acid–protein complexes (Citovsky and Zambryski, 1993). Nuclear export of a 75S premessenger ribonucleoprotein particle in *Chironomus tentans*, for example, initiates exclusively at the 5′ end of the RNA (Mehlin et al., 1992). Thus, it is possible that Agrobacterium has evolved to use the functionally different VirE2 and VirD2 NLSs to minimize competition between them for the plant cell receptors and thereby determine the polarity of the transport.

The function of VirE2 in promoting the T-strand nuclear import is consistent with previous observations that (1) tumorigenicity of an avirulent VirE2 mutant of Agrobacterium is

restored when inoculated on a VirE2-expressing transgenic plant (Citovsky et al., 1992b), and (2) coinoculation with an avirulent strain of Agrobacterium lacking T-DNA but expressing VirE2 complements the tumorigenicity of another avirulent strain lacking VirE2 but carrying the wild-type T-DNA, suggesting that VirE2 can enter the plant cell independent of the T-DNA (Binns et al., 1995). Furthermore, fewer T-strands have been shown to accumulate in the cytoplasm of plant cells infected with a VirE2-minus mutant of Agrobacterium, suggesting that VirE2 protects the T-strand from exonucleolytic degradation (Yusibov et al., 1994); this protective activity of VirE2 was also demonstrated in vitro (Citovsky et al., 1989). All of these observations strongly indicate that VirE2 forms a complex with the transported T-strand before nuclear import, supporting the idea that the T-strand is transported into the nucleus as a protein–ssDNA complex (T-complex).

Relevance to the General Mechanism of Nuclear Import of Nucleic Acids

In our model of the T-DNA nuclear import, VirE2 functions to bind the T-strand molecule and transport it across the nuclear membranes. Experimentally, the DNA transport function of VirE2 has not been demonstrated. We now describe this function by using microinjection of in vitro–formed complexes between ssDNA and purified VirE2s20. Our observations show that free DNA molecules microinjected into the oocyte cytoplasm do not enter the cell nucleus. They accumulate in the nucleus only when complexed with VirE2s20, a nuclear-targeted SSB. The VirE2s20–ssDNA complexes are most likely to be imported into the cell nucleus via a protein-specific pathway, because this import was inhibited by the excess of a synthetic bipartite NLS peptide. VirD2, which also contains an NLS but does not bind ssDNA, did not promote nuclear import, indicating that the NLS signals must be physically associated with the transported DNA molecule. Although the NLS signals of VirE2 may be plant specific, their biological function, that is, nuclear transport of DNA, is not. Thus, DNA nuclear import by formation of complexes between DNA and a specialized transport protein(s) may be relevant to many eukaryotic organisms. In addition, VirE2s20 may be used to deliver DNA into the cell nuclei efficiently, thereby improving the rate of transformation and producing homogenous rather than mosaic patterns of gene expression. Our model for protein-mediated nuclear import of nucleic acids is supported by the recent observations that (1) influenza virus nucleoprotein transports the viral genomic RNA into the cell nucleus in an in vitro system (O'Neill et al., 1995), and (2) the wild-type VirE2 actively transports ssDNA into the nucleus of the stamen hair cells of Tradescantia (Zupan et al., 1996).

METHODS

Protein Purification

We produced VirD2 and VirE2 in *Escherichia coli* BL21(DE3)pLysE with the T7 RNA polymerase expression system (Studier et al., 1990). The proteins were expressed as fusions with the first 11 amino acids of the gene 10 protein of the pET3 vector (Studier et al., 1990), as described previously (Citovsky et al., 1989; Howard et al., 1989), and purified as described for purification of the gene I protein of cauliflower mosaic virus (Citovsky et al., 1991). The VirE2s11 and VirE2s20 mutants of VirE2 were constructed by oligonucleotide-directed mutagenesis (McClary et al., 1989). The mutant proteins were then produced, purified, and fluorescently labeled as described for the wild-type VirE2.

Fluorescent Labeling of Proteins

Protein solutions (2 to 4 mg/mL) in 10 mM Hepes, pH 7.5, 100 mM NaCl, 10% glycerol (buffer H) were mixed in a 1:25 (w/w) ratio with tetramethylrhodamine-5-(and-6-)-maleimide (Molecular Probes, Inc.) and incubated for 2 hr at room temperature in the dark. After incubation, the labeled proteins were separated on SDS–polyacrylamide gels and electroeluted as described by Citovsky et al. (1989); this procedure resulted in >95% pure and biologically active protein preparations (Citovsky et al., 1989). The fluorescently labeled proteins were adjusted to the 4 mg/mL concentration, aliquoted, and stored at −70°C until use.

Fluorescent Labeling of DNA

Fluorescent DNA was obtained by polymerase chain reaction (PCR) amplification of an unrelated DNA fragment (a 717-bp promoterless sequence of the *Aequorea victoria* green fluorescent protein gene; Chalfie et al., 1994). The reaction was performed using the DeepVent DNA polymerase (New England Biolabs, Beverly, MA) in the presence of 0.4 mM dATP, dCTP, and dGTP, 0.3 mM dTTP, and 0.02 mM of rhodamine-conjugated dUTP (Boehringer Mannheim). After 32 cycles of amplification (2 min at 94°C, 5 min at 55°C, and 3 min at 72°C), the labeled DNA was separated from the unincorporated label on NucTrap push-columns (Stratagene).

Formation of Protein–ssDNA Complexes

Fluorescently labeled DNA (1 μL of 1 mg/mL solution) was denatured for 5 min at 95°C in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. The resulting single-stranded DNA (ssDNA) was incubated for 10 min at 4°C with saturating amounts (10 μg) of unlabeled VirE2 or VirE2s20 proteins, and the protein–ssDNA complexes were injected immediately into Xenopus oocytes.

Gel Mobility Shift Assay

The indicated amounts of the purified VirE2, VirE2s11, or VirE2s20 proteins were incubated for 10 min at 4°C in 20 μL of buffer H with

0.025 μg of the end-labeled ssDNA (717-bp fragment of the green fluorescent protein gene DNA; Chalfie et al., 1994). To produce ssDNA, the radioactively labeled probe was denatured for 5 min at 95°C. After incubation, protein–ssDNA complexes were resolved on a 4% native polyacrylamide gel, as described by Citovsky et al. (1989).

Microinjection of Drosophila Embryos

Drosophila embryos were dechorionated, affixed to a coverslip, and air dried for 5 to 7 min. The coverslips were then transferred to the stage of an inverted microscope, and the embryos were microinjected with fluorescently labeled protein. The distribution of the label within the microinjected embryos was monitored using a Bio-Rad MRC 600 laser scanning confocal attachment and a Nikon (Melville, NY) Diaphot inverted microscope.

The fluorescent staining patterns shown in Figures 1 to 3, 5, and 7 represent at least four independent experiments. In each experiment, 10 to 20 individual Drosophila embryos or Xenopus oocytes (see below) were microinjected, and the images were recorded 30 min after injection (except for the kinetic experiments in Figure 2, in which the nuclear import was recorded at 1-min intervals). During coinjection experiments, the concentration of unlabeled synthetic peptides corresponding to the VirD2 nuclear localization signal (NLS) and VirE2 NSE2 sequences was 5 mg/mL (coinjection with VirD2) or 4 mg/mL (coinjection with VirE2 and VirE2s20). These concentrations corresponded to an ~30-fold molar excess of peptide in a 1:1 (v/v) peptide-to-protein coinjection mixture. The amino acid sequence of VirD2 NLS and VirE2 NSE2 is shown in Figure 4.

Microinjection of Xenopus Oocytes

Full-grown Xenopus oocytes (stage VI) were obtained and microinjected with rhodamine-labeled protein into the cytoplasm at the equator of the oocyte or into the nucleus, as described by Kay (1991). Thirty minutes after injection of rhodamine-labeled proteins, DNA, or DNA–protein complexes, oocytes were fixed in methanol, cleared with benzyl benzoate:benzyl alcohol (2:1), and viewed under a confocal microscope, as described for Drosophila embryos. For isolation of the cell nuclei, the fixed and cleared oocytes were processed as described by Gall and Murphy (1991).

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