Basal Localization of the Presumptive Auxin Transport Carrier in Pea Stem Cells

Abstract. *By means of an indirect immunofluorescence technique with the use of monoclonal antibodies, the location of the presumptive auxin transport carrier of pea stem tissue was identified in the plasma membranes at the basal ends of parenchyma cells sheathing the vascular bundles. The results represent what is believed to be the first direct evidence for the hypothesized basal efflux carrier conferring polarity to auxin transport in plant stems.*

The plant growth regulator auxin acts to coordinate the growth and differentiation of subapical tissues in plants with the ongoing development of shoot apices. Auxin is synthesized in the apical meristems of shoots and in young leaves and moves in a polar fashion preferentially downward through the stem to the sites at which it exerts its numerous and varied effects. This basipetally polar auxin transport, first reported by Went in 1928 (*1*), has been shown to be an energy-requiring process, specific for auxins, with a velocity that varies from 5 to 15 mm per hour. The transport is to a large extent independent of the direction of gravity and is specifically inhibited by a diverse group of synthetic substances which includes naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) [for a review, see (*2*)]. Although all cells may transport auxin in coleoptiles (*3*), tissues of the vascular strands appear to be the major transport pathway in stems (*4–6*).

Although the phenomenon of polar auxin transport has been well characterized during the last 50 years, the mechanism by which the process occurs has not been elucidated. A currently attractive hypothesis (*7, 8*) is the chemiosmotic hypothesis of polar auxin transport, according to which a stem cell expends energy to maintain a *p*H gradient across its plasma membrane, with its cell wall more acidic than its cytoplasm. In the acidic environment of the cell wall, molecules of the native auxin, that is, indoleacetic acid (IAA) ($pK = 4.7$), will be in the undissociated form (IAAH). Since cells are more permeable to IAAH than to auxin anions (IAA^-), the undissociated form can readily enter the cell by diffusion. Once inside the cytoplasm, auxin molecules tend to dissociate to IAA^- in the higher prevailing *p*H, and in this form cannot freely equilibrate across the plasma membrane. As the concentration of IAA^- increases, movement out of the cell is thermodynamically favorable but can only occur via specific IAA^- carrier molecules that are hypothesized to lie only, or at a greater concentration, in the basal plasma membrane of the cell.

According to the chemiosmotic hypothesis, the polarity of auxin movement through a tissue is due to the basal localization of the anion carrier. Some previous experiments have supported the idea of a saturable auxin efflux carrier as the feature responsible for polarity of auxin movement, even though the location of the carrier was not established (*9*). Another report suggested, through indirect evidence, that "polar secretion" of auxin takes place preferentially at the basal ends of transporting cells (*10*). Yet no reports to date have provided specific evidence for the basal location of an actual protein that could be acting as an auxin anion carrier in polar auxin transport. In the study reported here we identified the location of the carrier by indirect immunofluorescence with monoclonal antibodies.

Monoclonal antibodies to the presumed auxin anion carrier were made by injecting a general pea membrane preparation into BALB/c mice. Tissue (15 g) from third internodes of 7-day-old etiolated pea seedlings (*Pisum sativum*, var. Alaska) was chopped with razor blades and ground for 2 minutes in a mortar and pestle in 15 ml of grinding medium (GM) [250 m*M* sucrose, 1 m*M* EDTA, 1 m*M* DTE (dithioerythritol), 0.1 m*M* $MgCl_2$, and 50 m*M* tris-mes, that is, tris(hydroxymethyl)aminomethane and 2-(*N*-morpholino)ethanesulfonic acid, *p*H 8].

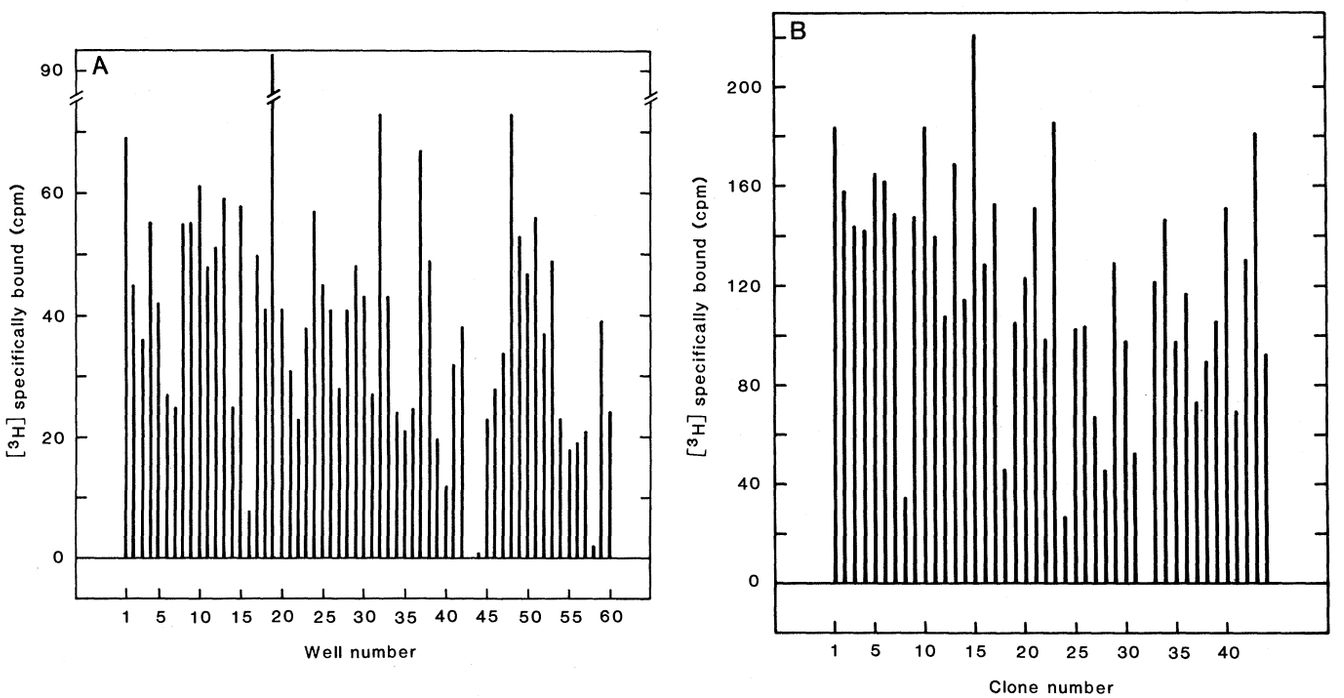

Fig. 1. Effects of antibody supernatant from (A) wells and (B) clones on specific binding of ^3H-labeled NPA to a membrane-bound site. Pea membranes were prepared and specific NPA binding was assayed as described previously (*22*), except that the total assay sample volume was 0.5 ml, and included 0.1 ml of antibody supernatant. Cells from well No. 16 in (A) were cloned in agarose to produce the 44 clones tested in (B).

The grindate was filtered through a fine nylon mesh, and the tissue remaining on the mesh was reground in a second 15-ml of GM and refiltered. The combined filtrates were centrifuged at 15,000*g* for 10 minutes. The supernatant of this spin was then centrifuged at 40,000*g* for 30 minutes. The resulting pellet was resuspended in 15 ml of resuspension medium (RM) [250 m*M* sucrose, 1 m*M* DTE, 0.5 m*M* $MgCl_2$, and 10 m*M* MOPS (morpholinopropane sulfonic acid), *p*H 6.5]. The resuspended membrane preparation was diluted with RM to a total protein concentration of 1 μg/μl. This preparation (200 μl) was injected three times into each mouse. Spleen cells from the immunized mice were fused with NS-1 plasmacytoma cells, and hybrid clones were selected in HAT (hypoxanthine, aminopterin, thymidine) tissue culture medium (*11*).

After the hybrid cells had grown for 20 days, we screened the culture supernatant from each well for the ability to inhibit NPA binding to membrane-bound sites (*12*) in pea epicotyl tissue. We did not use a specific auxin binding assay as the screening test since in any general membrane preparation from stem cells several different auxin binding sites are known to exist (*13*). Even in a plasma membrane–enriched fraction there are probably sites that can interact with auxin other than the anion efflux carrier [see discussion in (*14*)]. However, NPA has only one known binding site in plant stem cells, and that is believed to be on the plasma membrane (*12*). It also has only one well-documented effect in the same cells: a specific, strong inhibition of polar auxin transport that is thought to take place at the efflux site (*15, 16*). It is therefore likely that the NPA binding site is either identical to or interacting with the auxin anion carrier. We therefore used the NPA binding assay as our screening test for an antibody potentially marking the polar efflux carriers.

The results of our screening test for the supernatants in wells in one of our three plates are shown in Fig. 1A. Several wells contained antibodies that interfered with the NPA binding assay. We selected a well with one of the most inhibitory antibodies (No. 16) and cloned its cells in agarose. We then tested the resulting clones for their ability to inhibit NPA binding (Fig. 1B). Several clones produced antibodies that strongly inhibited NPA binding, and we used the culture fluid from these clones to identify the NPA binding sites in pea stem cells by indirect immunofluorescence.

Median longitudinal sections of third internode tissue from the etiolated pea seedlings were cut freehand with a single-edged razor blade from segments of fresh material. The sections were about 1

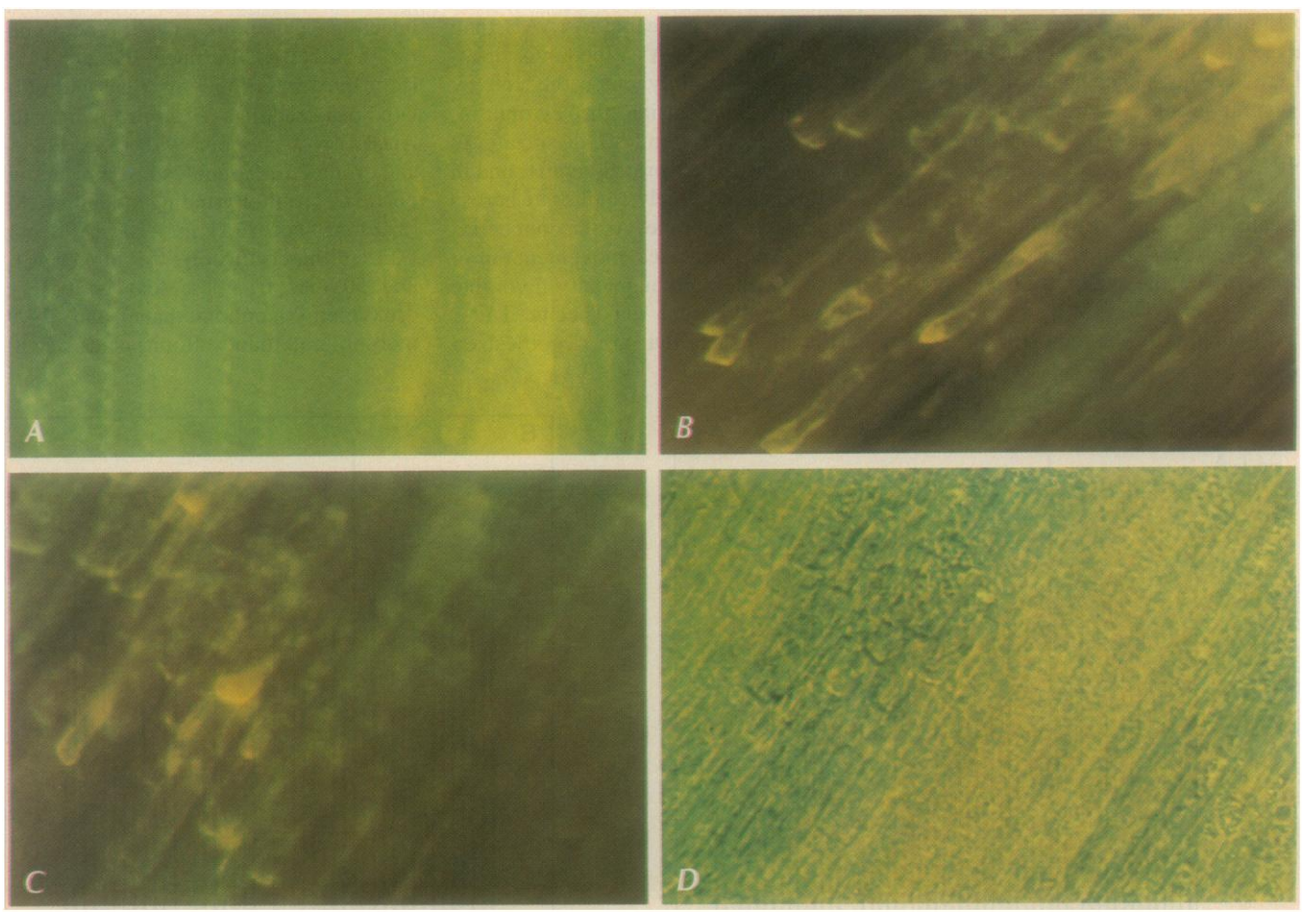

Fig. 2. Indirect immunofluorescence of pea stem tissue. Median longitudinal sections of third internode tissue of pea were incubated for 45 minutes at room temperature with clonal culture supernatants. The sections were rinsed three times in 0.5 percent bovine serum albumin in phosphate-buffered saline (PBSA), and further incubated, for 45 minutes, in fluorescein-conjugated rabbit antiserum to mouse immunoglobulin (Dako, Copenhagen; 1:100 in PBSA). The sections were then rinsed three times in PBSA, mounted in 50 percent glycerol, and observed by phase-contrast and fluorescence microscopy (×200). (A) Stem section incubated with clone No. 11 supernatant (inactive); view of area near vascular bundle. Basal end of section is at base of picture. Autofluorescing xylem elements are visible on left. The same results were obtained when sections were first incubated with monoclonal immunoglobulin G to GQ ganglioside. (B and C) Stem sections incubated with clone No. 24 supernatant (active); views of areas near vascular bundles. Basal end of each section is at lower left. In (B), a vascular bundle runs from the upper right to the lower left, showing a diffuse gray-green autofluorescence. In (C), the vascular bundle was just out of focus on the right-hand side of the picture. Similar labeling patterns were obtained with supernatants from clones Nos. 8, 18, and 32. (D) A phase-contrast view of the same stem section shown in (C). The end plates of some of the cells labeled in (C) are visible on the left.

cm in length, the width of the internode, and about 500 μm thick on one end tapering to about 20 μm in thickness at the other. The basal end of each section was identified with an angled razor cut. The sections were rinsed briefly in distilled water, incubated with monoclonal antibody supernatants, washed in phosphate-buffered saline solution, incubated with fluorescein-conjugated rabbit antiserum to mouse immunoglobulin G (Dako, Copenhagen), and observed under ultraviolet light.

Sections of control pea stems (Fig. 2A) that were exposed to antibodies inactive in inhibiting NPA binding in the screening assay showed a relatively strong blue-gray autofluorescence from xylem cell walls and a diffuse background fluorescence. When an active antibody was used, groups of cells (Fig. 2, B and C) were specifically labeled, showing strong fluorescence only at their basal ends. In experiments in which the stem cells were plasmolyzed (in 1*M* and 2*M* sucrose) after exposure to the fluorescent antibody, the bright fluorescence remained with the plasma membrane as it pulled away from the basal cell wall (data not shown). The U-shaped fluorescent zones in these cells therefore indicate a distribution of the NPA binding site that is primarily on the basal plasma membrane, but which also extends part way up the side membranes of the cell. It is important to note that not all cells in these sections were labeled. Groups of labeled cells were only observed when the plane of section passed through a vascular bundle in the internode and, when observed, were always in tissue that appeared to be sheathing or closely associated with the vascular bundles and composed of elongated parenchyma cells. Specific fluorescent labeling was never seen in pith or cortical parenchyma cells. When labeled cells were observed (in over 20 separate longitudinal sections) the specific fluorescence was, without exception, only seen at the basal ends of the cells.

To investigate possible interactions between the NPA receptor and auxin, we solubilized the NPA binding site from general pea membrane preparations and found that the specific NPA binding that we isolated was IAA-sensitive (Table 1). A similar IAA-sensitive, solubilized NPA binding was reported by Sussman and Gardner in Triton-treated corn coleoptile membranes (*17*). When we performed the NPA-IAA binding assay with these solubilized NPA binding sites in the presence of supernatant from either a clone (No. 11) that was inactive in inhibiting NPA binding in our screening assay (see Fig. 1B) or a clone (No. 8) that was an active inhibitor, we found that both specific NPA binding to the solubilized site and the IAA-sensitive component were inhibited by the active clone (Table 2). This indicates that (i) the NPA binding site recognized by the monoclonal antibody could interact with the plant growth regulator IAA and (ii) that interaction was substantially modified by application of the active antibodies. We thus located a protein that binds NPA and can interact with IAA, two characteristics expected of the auxin anion carrier described by the chemiosmotic hypothesis.

Aside from its immediate relevance to the chemiosmotic hypothesis of polar auxin transport, the basal localization of the NPA binding sites within each labeled cell is also interesting in relation to the use of NPA binding as a plasma membrane marker in cell fractionation studies (*18*). Several researchers have found a lack of exact agreement between NPA binding and other putative plasma membrane markers when assayed in the same density gradients (*19, 20*). The possibility that NPA binding marks only one domain within the plasma membrane has already been advanced (*18*), and the evidence presented here would tend to support that contention.

The results in Fig. 2, B and C, which

Table 1. Effects of unlabeled NPA and IAA on the binding of ^{3}H-labeled NPA to a solubilized receptor from a general pea membrane preparation. Pea membranes were prepared as described in the text except that they were resuspended, after the centrifugation at 40,000*g*, in RM containing 1 percent Triton X-100. They were then homogenized, incubated for 20 minutes on ice, and recentrifuged at 40,000*g* for 30 minutes. The resulting supernatant contained the solubilized NPA receptor and was used for the experiments in Tables 1 and 2 (the solubilized NPA receptor remained in the supernatant after additional centrifugation at 110,000*g* for 60 minutes). Binding to the solubilized NPA receptor was assayed with an Amicon MPS-1 micropartition system with YMT membranes (Amicon). Total assay sample volume was 0.5 ml and specific NPA binding was assayed at the concentrations described previously (*22*). The sample volume was centrifuged completely through the YMT membrane by mounting the MPS-1 assemblies in a refrigerated centrifuge and spinning at 1600*g* for 50 minutes. Dry membranes, retaining the solubilized receptor-ligand complex, were removed and counted in Scintisol (Isolab) in a liquid scintillation counter. Numbers in parentheses are standard error of the mean for triplicate samples.

Treatment	Radioactivity (count/min)		
	Bound [^{3}H]NPA	Specific NPA binding (A − B)	IAA-sensitive NPA binding (A − C)
A. $10^{-9}M$ [^{3}H]NPA	1432 (59)	466	
B. $10^{-9}M$ [^{3}H]NPA plus $10^{-5}M$ NPA	966 (46)		233
C. $10^{-9}M$ [^{3}H]NPA plus $10^{-5}M$ IAA	1199 (30)		

Table 2. Comparison of the effects of active and inactive antibodies on specific and IAA-sensitive NPA binding to a solubilized receptor. Solubilized receptor and [^{3}H]NPA binding assay were prepared as described in Table 1 except that the 0.5-ml assay volume was composed of 0.4 ml of solubilized NPA receptor preparation and 0.1 ml of antibody supernatant. Numbers in parentheses are standard error of the mean for triplicate samples.

Treatment	Radioactivity (count/min)		
	[^{3}H]NPA bound	Specific NPA binding (A − B)	IAA-sensitive NPA binding (A − C)
Clone 11 supernatant (inactive)			
A. $10^{-9}M$ [^{3}H]NPA	1704 (126)	752	
B. $10^{-9}M$ [^{3}H]NPA plus $10^{-5}M$ NPA	952 (17)		526
C. $10^{-9}M$ [^{3}H]NPA plus $10^{-5}M$ IAA	1178 (117)		
Clone 8 supernatant (active)			
A. $10^{-9}M$ [^{3}H]NPA	1494 (252)	353	
B. $10^{-9}M$ [^{3}H]NPA plus $10^{-5}M$ NPA	1141 (19)		−120
C. $10^{-9}M$ [^{3}H]NPA plus $10^{-5}M$ IAA	1614 (168)		

indicate a distribution of the presumptive auxin carriers part way up the sides of the cells in addition to the base would easily allow a lateral component to auxin transport if either only one side's carriers were activated or if basal and "other side" carriers were inhibited. Such differential activation of the auxin anion carriers is a concept already being incorporated into theoretical models of auxin transport in, for example, the geotropic response of stems (*21*).

The association of the labeled cells with vascular bundles in pea stems appears to be in general agreement with recent evidence from peas (*6*) that indicated a preferred auxin transport pathway within the vascular cylinder, specifically in cambium, procambium, and phloem initial cells. At this point, however, it is impossible for us to exclude the possibility that differential fluorescent labeling of cell and tissue types within our sections is due solely to such factors as differential penetration of cell walls by the immunoglobulin G antibody or a concentration of NPA binding sites in nonlabeled cells (or nonlabeled parts of basally labeled cells) that is too low to allow the development of a clear fluorescent signal. These alternatives deserve further investigation.

To our knowledge, this is the first report of monoclonal antibodies used to label plant tissue. Such antibodies are useful in that they can recognize a single antigenic site. Thus, one need not have a purified protein in order to obtain antibodies which specifically bind to it. We have obtained monoclonal antibodies that recognize an antigenic determinant of the NPA receptor in peas and have used them to identify that receptor at the basal ends of a population of pea stem parenchyma cells. In view of previous evidence linking NPA action to auxin efflux from cells and our present finding that the NPA binding site recognized by the monoclonal antibody used in our fluorescent localization studies can interact with auxin, these results strongly suggest that the auxin anion carrier of the chemiosmotic hypothesis is located at the basal plasma membrane of transporting cells.

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23. We thank G. Flickinger, P. Kloeckner, W. Kirby, and J. Halprin for technical assistance. Supported by grant PCM 81-04488 from the National Science Foundation (M.J.) and NIH grant HD-15032 (S.F.G.).

4 March 1983; revised 15 April 1983

Nuclear Transplantation in the Mouse Embryo by Microsurgery and Cell Fusion

Abstract. *Nuclear transplantation in the mouse embryo was achieved by using a method that combines microsurgical removal of the zygote pronuclei with the introduction of a donor nucleus by a virus-mediated cell fusion technique. Survival of embryos was greater than 90 percent in tests of this procedure. The embryos developed to term at a frequency not significantly different from that of nonmanipulated control embryos. Because nuclei and cytoplasm from genetically distinct inbred mouse strains can be efficiently interchanged, this procedure may be useful in characterizing possible cytoplasmic contributions to the embryonic and adult phenotype.*

Nuclear transplantation studies in the amphibian embryo have provided valuable information about the possible restriction of nuclear potential during development (*1*). Similar experiments in the mammalian embryo are hindered by the small size of the embryo and its sensitivity to microsurgical manipulation. Despite these obstacles, transplantation of donor nuclei obtained from inner cell mass cells was recently reported in the mouse embryo (*2, 3*). In those experiments, however, many of the manipulated embryos were lost because the plasma membrane was disrupted by a micropipette. We report a nuclear transplantation technique that avoids this loss by not requiring penetration of the embryo's plasma membrane with a micropipette. When pronuclei from a second one-cell stage embryo served as the nuclear donor, almost all embryos receiving this material survived the procedure and developed to term at a frequency comparable to that of unmanipulated control embryos.

Mouse embryos were incubated before and during microsurgery in cytochalasin B (*2–5*) and Colcemid. The embryo was secured by a holding pipette and the zona pellucida was penetrated with an enucleation pipette (Fig. 1). Penetration of the plasma membrane was avoided and the pipette was advanced into the embryo at a point adjacent to a pronucleus. Upon aspiration, a small portion of ovum plasma membrane and surrounding cytoplasm was drawn into the pipette, followed by the pronucleus. The pipette, now containing an entire pronucleus, was then moved to a point adjacent to the second pronucleus and the latter was similarly aspirated. As the enucleation pipette was withdrawn, a cytoplasmic bridge could be seen extending from the pronuclei in the pipette to the embryo (Fig. 1A). With continued withdrawal of the pipette, this bridge stretched to a fine thread and pinched off (Fig. 1B). The pipette, which now contained the membrane-bound pronuclei (pronuclear karyoplast), was moved to a

Table 1. Efficiency of the nuclear transplantation technique.

Genotype	Enucleation*	Karyoplast injection†	Fusion‡
C3H/HeJ	24 of 26	24 of 24	23 of 24
C57BL/6J	35 of 35	34 of 35	34 of 34
ICR	11 of 12	10 of 11	10 of 10
Total (%)	70 of 73 (96)	68 of 70 (97)	67 of 68 (99)

*Number of embryos surviving microsurgical removal of both the male and female pronuclei per total number of embryos. †Number of pronuclear karyoplasts surviving injection into the perivitelline space of the recipient embryo per total number of karyoplasts injected. ‡Number of pronuclear karyoplasts fusing with the recipient embryo per total number of karyoplast-injected embryos.