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Homology-dependent Gene Silencing in *Paramecium*

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Microinjection at high copy number of plasmids containing only the coding region of a gene into the *Paramecium* somatic macronucleus led to a marked reduction in the expression of the corresponding endogenous gene(s). The silencing effect, which is stably maintained throughout vegetative growth, has been observed for all *Paramecium* genes examined so far: a single-copy gene (*ND7*), as well as members of multigene families (centrin genes and trichocyst matrix protein genes) in which all closely related paralogous genes appeared to be affected. This phenomenon may be related to posttranscriptional gene silencing in transgenic plants and quelling in *Neurospora* and allows the efficient creation of specific mutant phenotypes thus providing a potentially powerful tool to study gene function in *Paramecium*. For the two multigene families that encode proteins that coassemble to build up complex subcellular structures the analysis presented herein provides the first experimental evidence that the members of these gene families are not functionally redundant.

INTRODUCTION

The ciliate *Paramecium* is a unicellular organism that displays differentiated traits generally restricted to metazoans. Among these are the regulated exocytosis of trichocysts, secretory granules thought to be involved in defense against predators (for review Adoutte, 1988), and a developmental program that ensures the heredity of a polarized and asymmetric cortical pattern at cell division (Iftode *et al.*, 1989; Jerka-Dziadosz and Beisson, 1990). Common to regulated secretion and cortical morphogenesis is the elaborate architecture of the constituent subcellular structures, and both the trichocyst crystalline core and a number of cytoskeletal arrays are known to be built up from sets of closely related polypeptides (Tindall *et al.*, 1989; Garreau de Loubresse *et al.*, 1991; Sperling *et al.*, 1991; Coffe *et al.*, 1996). We have previously shown that the trichocyst matrix proteins (TMPs)[1] and the polypeptides that form the innermost cytoskeletal network of the *Paramecium* cortex (the infraciliary lattice, ICL) are encoded by multigenic families. The TMP and the ICL gene families, estimated to contain ~100 and ~20 members, respectively, are both organized in subfamilies that code for distinct proteins; within each subfamily several genes code for almost identical polypeptides (Madeddu *et al.*, 1995, 1996). Most strikingly, all of these genes seem to be coexpressed and thus mixtures of related polypeptides are produced that coassemble. We have suggested that in *Paramecium* these multigene families have been generated to respond to functional requirements (Madeddu *et al.*, 1995, 1996).

Studies of transgenic organisms have led to the discovery of a diverse set of phenomena in plants, fungi, and *Drosophila*, by which extra nuclear copies of a gene cause reduced expression of some or all of the copies of that gene (for reviews Baulcombe, 1996; Bingham, 1997; Depicker and van Montagu, 1997; Stam *et al.*, 1997). Despite the common outcome, usually termed gene silencing or cosuppression, there seems to be considerable heterogeneity in the underlying molecular mechanisms. In no case is the mechanism completely understood; however, transgene-induced silencing can involve DNA methylation (Malagnac *et al.*, 1997), the establishment of stably repressed chromatin (Pal-Bhadra *et al.*, 1997), or RNA-mediated RNA deg-

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[1] Abbreviations used: TMP, trichocyst matrix protein; ICL, infraciliary lattice.

radation (Cogoni and Macino, 1997; Metzlaff *et al.*, 1997).

In the present report, we describe the characteristics of a phenomenon in *Paramecium* of specific repression of gene expression achieved by the introduction of many copies of the coding region of a target gene, without any flanking sequences, into the somatic nucleus. Our experiments involved members of the TMP and the ICL multigene families and a single-copy gene, *ND7*, which was recently cloned by functional complementation of the hypomorphic allele *nd7–1* (Skouri and Cohen, 1997). *ND7* is required for exocytotic membrane fusion and trichocyst release, a phenotype that lends itself to quantitative evaluation. We found that microinjection of coding sequences impaired expression of the corresponding endogenous gene copies, creating mutant phenotypes defective in the cellular structures built up from the products of the silenced genes. Microinjection of the coding regions of genes belonging to different TMP subfamilies specifically reduced the expression of most or all subfamily members, yielding phenotypically distinct mutant trichocysts. These results provide direct support for the hypothesis (Madeddu *et al.*, 1995, 1996) that these families of coexpressed genes evolved to assure microheterogeneity among coassembling structural proteins necessary for the edification of geometrically complex dynamic subcellular assemblies. Microinjection of the coding region of the *ND7* gene allowed us to obtain the same exocytosis-deficient phenotype conferred by the *nd7–1* hypomorphic allele. The existence in a ciliate of a phenomenon potentially related to gene silencing in higher eukaryotes and fungi could help explore the important question of whether or not the various silencing phenomena all derive from a single ancestral system established early in evolution (Bingham, 1997).

MATERIALS AND METHODS

Cells and Culture Conditions

Wild-type cells were *Paramecium tetraurelia* strain d4–2. Two *P. tetraurelia* secretory mutant strains were also used in these experiments: tam8 cells contain morphologically normal trichocysts free in the cytoplasm, unable to attach to the plasma membrane (Beisson and Rossignol, 1975), and nd7 trichocysts are docked at their specific cortical sites but cannot undergo exocytosis (Lefort-Tran *et al.*, 1981). Cells were grown at 27°C in Scotch grass infusion, inoculated with *Enterobacter aerogenes* and supplemented with 0.4 μg/ml β-sitosterol (Sonneborn, 1970).

Plasmid Preparation and Microinjection

DNA fragments were generated by PCR amplification; the templates used were recombinant plasmids containing the selected sequences, obtained as previously described (ICL1a and ICL1b genes, Madeddu *et al.*, 1996; T1b and T4a genes, Gautier *et al.*, 1996; *ND7* gene, Skouri and Cohen, 1997). PCRs (50 μl) contained 100 pmol of each primer, all four dNTPs (each at 0.2 mM), and 2 U of *Taq* DNA polymerase (Boehringer). Reactions were carried out for 30 cycles of denaturation at 90°C for 30 s, annealing at 48°C for 45 s, and extension at 72°C for 1 min 30 s.

The oligonucleotide primers designed to amplify DNA fragments corresponding to coding regions were as follows, with the sense primer being the first in each pair: 5′-GGCACGAAGAGGATAGTAACCACCACCC-3′ and 5′-GCAAAGGTCTTTTTTGTCATAATGTTGTAG-3′ (ICL1); 5′-ATGTATAAATTAGCAGTCTGCACATTGC-3′ and 5′-TCAAAATGCTCCCTTGAGTTGGGATTTG-3′ (T1b); 5′-ATGGCTAGATCATTACAAATATTGGC-3′ and 5′-TCAAAATACTTCTTCTCTGACTTGGAGG-3′ (T4a); 5′-ATGAGAAAAATAATATAATTATTG-3′ and 5′-ATGACAGTAGATTCGTTTC-3′ (ND7).

Oligonucleotide primers used for amplification of the functional *ND7* gene, consisting of the entire coding region, 157 bp of upstream, and 249 bp of downstream flanking sequence, were as follows: 5′-AATGGAAATATAATTCATC-3′ and 5′-CTAAATACAATTATTAGGG-3′.

The amplification products were cloned either into the *Sma*I site of the pUC18 plasmid (ICL1, T1b, and T4a sequences) or, for *ND7* sequences, into pTAg vectors (R & D Systems, Minneapolis, MN), according to standard protocols (Sambrook *et al.*, 1989). CsCl-purified plasmid DNA was linearized within the vector sequence with *Ssp*I (ICL1 sequences), *Sca*I (T1b and T4a sequences), or *Sma*I (*ND7* sequences) and then extracted with phenol. After precipitation with ethanol, DNA was resuspended in water at 5–10 mg/ml. The p201ND7 plasmid (Skouri and Cohen, 1997), containing the wild-type *ND7* gene, was a kind gift of J. Cohen.

Young cells (five fissions after autogamy) were transferred to Dryl's buffer (2 mM sodium citrate, 1 mM NaH_2PO_4, 1 mM Na_2HPO_4, 1.5 mM $CaCl_2$; Dryl, 1959) supplemented with 0.2% bovine serum albumin. Cells were observed under a film of mineral oil (Nujol) with a Nikon inverted phase-contrast microscope, and DNA (approximately 5 pl) was delivered to the macronucleus, by using a Narishige micromanipulator device and an Eppendorf Transjector 5246.

Evaluation of Exocytotic Activity

The capacity of microinjected cells to secrete their trichocysts was analyzed by treatment with the fixing secretagogue picric acid, according to Pollack (1974). Cells were individually transferred to drops of a saturated solution of picric acid: discharged trichocysts remain attached to the cells, so that exocytosis is easily monitored under dark-field light microscopy at low magnification. Exponentially growing wild-type cells secrete all of their approximately 1000 docked trichocysts, which appear as a dense halo surrounding the cells.

Immunofluorescence

Immunofluorescence experiments were carried out as previously described (Garreau de Loubresse *et al.*, 1988). Briefly, after a 2-min permeabilization in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH6.9) containing 1% Triton X-100, cells were fixed in 2% paraformaldehyde; incubations with the primary and the secondary FITC antibody (Jackson ImmunoResearch Labs, West Grove, PA) were carried out in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.01% Tween 20, 5 mM $CaCl_2$, and 3% bovine serum albumin.

The anti-centrin monoclonal antibody 20H5 (Sanders and Salisbury, 1994), generously provided by J. Salisbury (Mayo Clinic, Rochester, MN), was used at a 1:500 dilution. A polyclonal antiserum directed against the TMPs (Gautier *et al.*, 1994) was used at a 1:1000 dilution.

Gel Electrophoresis and Western Blots

To prepare whole cell lysates, cell cultures were centrifuged, and the cell pellets were immediately mixed (vol/vol, final concentration, 10^6 cells/ml) with boiling 5% SDS, in the presence of protease

inhibitors (phenylmethylsulfonyl fluoride at 50 μg/ml, leupeptin at 5 μg/ml). SDS-PAGE fractionation was performed on 13% polyacrylamide gels (Laemmli, 1970); proteins were electroblotted to nitrocellulose membranes, and immunoreactions were carried out as previously described (Klotz *et al.*, 1997) with the anti-centrin 20H5 antibody (1:5000) and an anti-β-tubulin monoclonal antibody (1:2000; Amersham, Bucks, United Kingdom).

DNA Preparation and Southern Blots

DNA was extracted from exponential-phase cultures 20–25 fissions after injection according to Duharcourt *et al.* (1995), after the phenotypes of the cultures had been verified by the appropriate technique, immunofluorescent staining, and/or determination of exocytotic capacity. The extracted DNA was then fractionated by electrophoresis on 1% agarose gels. After transfer to Hybond-N^+ filters (Amersham), hybridizations were carried out according to Church and Gilbert (1984), at 60°C. The membranes were then washed at the same temperature with decreasing concentrations of SSC, in the presence of 0.1% SDS as follows: 2× SSC for ~30 min, followed by 0.2× SSC for 30–45 min (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.2; Sambrook *et al.*, 1989). Images were obtained by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Dot Blot Analysis

To determine the amount of transforming DNA in different clonal cell populations, duplicate aliquots of 50 cells were isolated manually and transferred to 400 μl of 0.8 N NaOH containing 250 mM EDTA. The cell lysates were incubated for 30 min at 65°C, cleared of debris by centrifugation at 10,000 × *g*, and loaded on Positive membranes (Appligene, Illkirch, France) by using a home-made dot-blot apparatus. After loading, the membranes were left in contact with 0.4 N NaOH for 15 min, washed in 2× SSC, and subsequently treated as described for Southern blots. Duplicate samples of uninjected control cells and a range of plasmid dilutions were included on each blot for calibration. Hybridization was quantified by using a PhosphorImager and ImageQuant software (Molecular Dynamics). All values are given as the average of the duplicate determinations.

RNA Preparation and Northern Blots

Total RNA was prepared essentially according to the method of Chomczynski and Sacchi (1987), except that the cells were lysed by vortex mixing in the presence of glass beads. RNA (15–20 μg/lane) was fractionated on formaldehyde-1.3% agarose gels and transferred to Hybond-C extra filters (Amersham). Hybridizations were carried out at 48°C in 6× SSC, 2× Denhardt's solution, and 0.1% SDS (Sambrook *et al.*, 1989); the filters were then washed and imaged as described for Southern blots.

Preparation of Radioactive Probes

Probes were generated by PCR amplification of cloned DNA fragments in the presence of [α-^{32}P]ATP (Amersham), as previously described (Madeddu *et al.*, 1995). The sense primers were the same used for the amplification of the coding regions, given above. The antisense oligonucleotide primers used were as follows: 5′-GATATCGGCTTTAGAATCTC-3′ (ICL1 probe), 5′-CAACTCTCAARTTATTGAAGGC-3′ (T1 probe), and 5′-CTTATCTTTCTTCTGTGGC-3′ (T4 probe).

For the ND7 probe, the sense primer was 5′-AGTAGAAGAGTTGTTATTG-3′; the antisense primer was the same used for amplification of the coding region.

The pUC18 probe (corresponding to the entire vector sequence) was prepared by using a random primers DNA labeling system kit (Life Technologies, Gaithersburg, MD).

RESULTS

Microinjected DNA Is Maintained in an Episomal State

Like all ciliates, *Paramecium* presents nuclear dimorphism. Each cell contains a transcriptionally inactive diploid micronucleus, involved in sexual processes (conjugation and autofertilization, or autogamy), and a somatic macronucleus, which is responsible for gene expression during vegetative growth. The macronucleus is highly polyploid (~800n in *P. tetraurelia*), divides amitotically, and consists of linear acentric chromosomes of 50–800 kb in size. During sexual events, the old macronucleus is degraded and replaced by a new one, derived from the germ-line micronucleus through extensive programmed DNA rearrangements: chromosome fragmentation, amplification, telomerization, and excision of thousands of germline–specific elements (for review Prescott, 1994). Transformation by direct microinjection of DNA into the somatic macronucleus is well established in *Paramecium*. Exogenous DNA molecules of various sources and sequences have been shown to be stably maintained throughout the vegetative cell cycle as linearized and telomerized pseudochromosomes, which can replicate autonomously in the absence of any identified replication origin. The injected DNA is lost at autogamy, along with the macronuclear chromosomes (Godiska *et al.*, 1987; Gilley *et al.*, 1988; Bourgain and Katinka, 1991). This phenomenon has generally been studied in the context of complementation experiments: expression of the microinjected sequences was evaluated in cells in which the functional product of the corresponding endogenous gene was absent due to choice of geographic strain (Meyer, 1992), deletion (Godiska *et al.*, 1987; Gilley *et al.*, 1988; Martin *et al.*, 1994), or mutation (Kanabrocki *et al.*, 1991; Haynes *et al.*, 1996; Skouri and Cohen, 1997).

In this study, plasmids containing different gene sequences (presented schematically in Figure 1) were microinjected at high copy number into the somatic macronucleus of *Paramecium* cells physiologically expressing their wild-type endogenous homologues; the phenotypes of transformed cells were followed through vegetative growth and characterized as detailed below for each of the constructs. The fate of the microinjected plasmids was in each case examined by Southern blot analysis of undigested DNA from the clonal descendants of the microinjected cells. A typical experiment, carried out with a clone transformed with pICL1b, is shown in Figure 2. A series of bands significantly smaller than the 50- to 800-kb macronuclear chromosomes and corresponding to multimers ($1 \leq n \leq 5$) of the microinjected plasmid were detected, using either a gene-specific (ICL1) or a plasmid-specific (pUC18) probe (Figure 2, lanes b and d). This pattern indicates that the vast majority of the injected

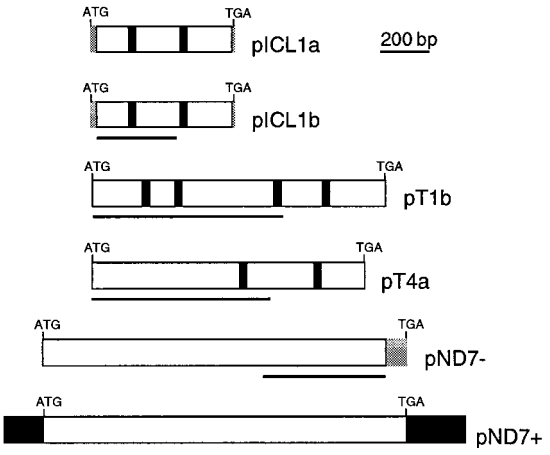

Figure 1. Maps of *Paramecium* gene sequences used in microinjection experiments. All sequences were cloned in plasmid vectors as described in MATERIALS AND METHODS. Open boxes, coding sequences; solid boxes, flanking or intron sequences; shaded boxes, coding sequences not included in the construct. Regions used as probes for hybridization are represented as a solid line; it is important to note that the ICL1b, T1b, and T4a probes are subfamily specific: they hybridize, respectively, with all members of the ICL1, T1, and T4 gene subfamilies on Southern blots (Madeddu *et al.*, 1995, 1996). The pND7+ plasmid insert is derived from p201ND7 previously described by Skouri and Cohen (1997).

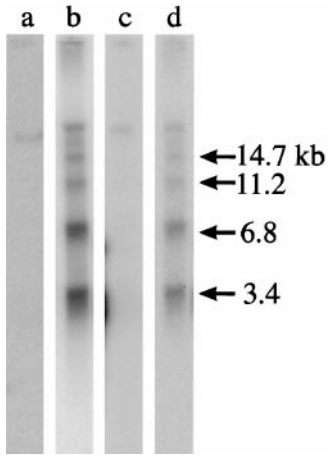

Figure 2. Maintenance of microinjected plasmid DNA. Total DNA was extracted from control uninjected nd7 cells (lane a), cells of a vegetative clone derived from a cell microinjected with pICL1b (lanes b and d), and cells of a postautogamous line derived from the pICL1b transformed clone (lane c). Undigested DNA was fractionated (4–8 μg/lane) on a 1% agarose gel by contour-clamped homogeneous electric field (CHEF) electrophoresis (9 V/cm; switch time, 0.1 s; angle, 120°; run time, 3 h), using a CHEF-DR III apparatus (Bio-Rad, Richmond, CA), and transferred to nitrocellulose. The same filter was sequentially hybridized with the ICL1 (lanes a–c) and the pUC18 (lane d) specific probes. The 50- to 800-kb *Paramecium* macronuclear chromosomes are not resolved in these experimental conditions: the weak signals detected in control and postautogamous cells most likely arise from hybridization with the endogenous ICL1 genes.

DNA is replicated as free linear molecules. The multimers arise from intramolecular concatenation, a common event after DNA injection (Bourgain and Katinka, 1991), and the smeared appearance of the bands reflects the variable length of the added telomeric repeats. As expected, no such bands were observed in uninjected control cells (Figure 2a) or in cells of a postautogamous clone (Figure 2c): the transforming DNA is lost at autogamy, when the macronucleus breaks down (Godiska *et al.*, 1987). Densitometric analysis of autoradiograms allowed us to estimate that the transforming DNA was maintained at 40 to 200 haploid genome equivalents in our experiments.

Cells Microinjected with ICL1 Coding Sequences Present a Defective ICL

The ICL is a Ca^{2+}-modulated contractile fibrous array that underlies the whole cell surface, visualized by specific immunofluorescent staining as a continuous network of polygonal meshes (Figure 3a). The ICL is essentially composed of a family of biochemically and immunologically related polypeptides (Garreau de Loubresse *et al.*, 1988, 1991). For one of them (ICL1), we have recently characterized four nearly identical coexpressed genes (Madeddu *et al.*, 1996; Vayssié *et al.*, 1997) that code for centrins, highly conserved EF-hand Ca^{2+}-binding proteins (for review Salisbury, 1995).

Plasmids containing DNA fragments corresponding to the coding region of two of these genes, without flanking regulatory sequences (pICL1a and pICL1b, see Figure 1), were used for microinjection experiments. To facilitate the screening of successfully transformed cells, the experiments were carried out not in wild-type cells but rather in cells of an exocytosis mutant, nd7–1. A plasmid bearing the complete wild-type *ND7* gene (Skouri and Cohen, 1997; pND7+ in Figure 1) was coinjected at a 1:1000 ratio. Rescue of the mutant phenotype through complementation (Skouri and Cohen, 1997) was evaluated by visually monitoring the exocytotic activity induced by treatment with picric acid, as described in MATERIALS AND METHODS.

Clonal descendants of 12 of 13 cells microinjected with pICL1a and of 9 of 12 cells microinjected with

Figure 3 (facing page). Phenotypic characterization of cells transformed with ICL1 coding sequences. (a) Immunolabeling of an uninjected nd7 cell by the anti-centrin antibody 20H5. The ICL is formed of irregular polygonal meshes of various sizes; the regionalized differences in their arrangement reflect the polarized and asymmetrical organization of the *Paramecium* cortex. (b and c) Immunofluorescence images showing clonal descendants of pICL1b-microinjected cells, with total or partial disorganization of the ICL network. pICL1a-transformed cells presented similar phenotypes.

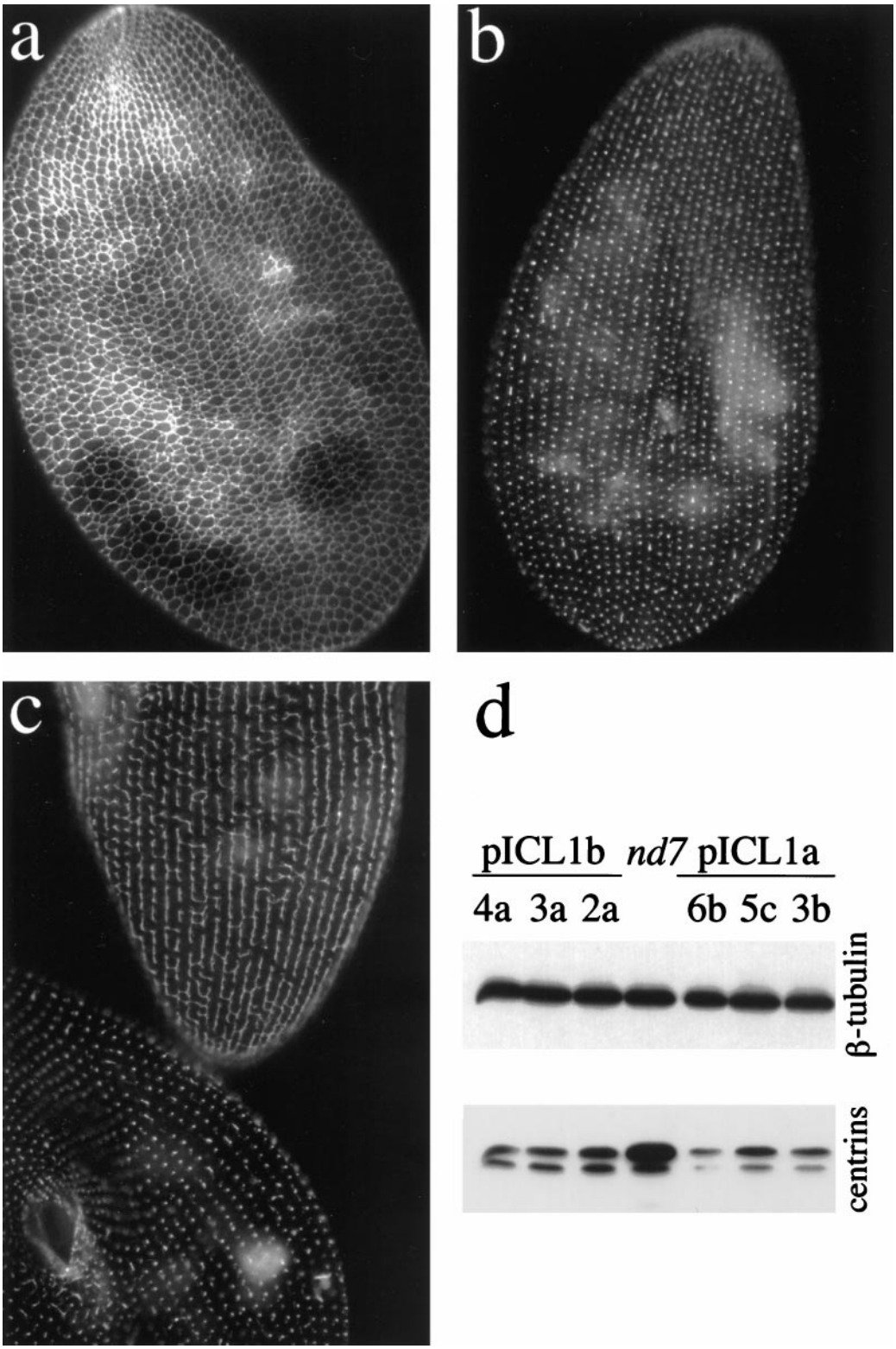

Figure 3 (cont). Magnification, 700×. (d) Western blot of total cellular proteins from cells of three pICL1a- and three pICL1b-transformed clones, compared with uninjected (nd7) cells. The same blot was treated with the 20H5 antibody (bottom) and with an antibody directed against β-tubulin (top) as a control for the amount of total protein in each sample.

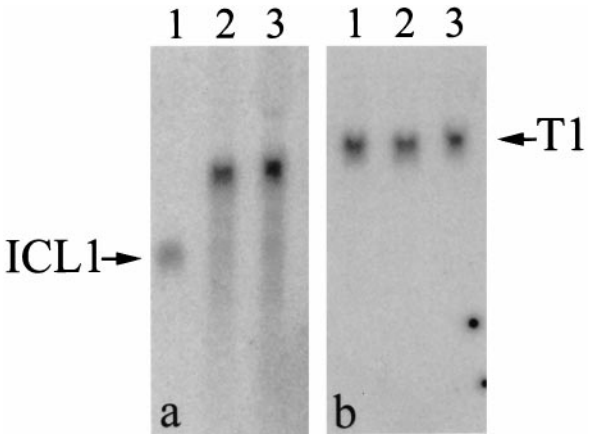

Figure 4. Northern blot analysis of pICL1b transformants. Total cellular RNA was prepared from uninjected nd7 cells (lane 1) and from two clones of pICL1b-transformed cells: clone 3a (lane 2) and clone 4a (lane 3). The same filter was hybridized sequentially with the ICL1b probe (a) and the T1b probe (b). The position of ICL1 mRNA (~0.85 kb) is indicated. T1 mRNA migrates at ~1.4 kb (Madeddu *et al.*, 1995).

pICL1b recovered exocytotic capacity two or three cell divisions after injection. The transformed cells displayed normal growth rate (four or five divisions/d). However, immunofluorescent centrin staining (five to eight fissions after microinjection) revealed a dramatic disorganization of the ICL. Total or partial disassembly of the ICL network was observed in all clones examined: only tufts of immunofluorescent material localized around the ciliary basal bodies (Figure 3, b and c, lower cell) or short stretches of polygonal meshes (Figure 3c, upper cell) were visible. The ICL-deficient phenotype was characteristic of all cells within each transformed clone. Similar results were obtained when pICL1a or pICL1b plasmids were microinjected into wild-type cells. Surprisingly, the absence of this massive contractile cytoskeletal network has no apparent effect on cellular growth or form: the ICL seems to be dispensable to the cell.

Although we have not systematically investigated the DNA sequences necessary to generate an ICL-deficient phenotype, microinjection experiments were also carried out with a plasmid containing only the 5′ half of the ICL1b coding region (exactly corresponding to the ICL1 probe, see Figure 1). When analyzed by immunocytochemistry, the ICL of clonal descendants of 10 of 13 microinjected cells still appeared abnormal, although the degree of disorganization of the network was less severe than that observed in experiments with the larger pICL1a and pICL1b constructs: the meshes were enlarged and many of the polygons were incomplete (our unpublished observations).

Deficient ICL Correlates with Reduced ICL1 Protein and mRNA Levels

Total cellular proteins from several clones microinjected with either pICL1a or pICL1b were separated by SDS-PAGE and analyzed by Western blot. The transformed cells had significantly lower levels of the polypeptides recognized by the anti-centrin antibody than uninjected control nd7 cells (Figure 3d). The degree of reduction, moreover, appeared to correlate with the severity of the phenotypic effects detected by immunofluorescence.

Steady-state ICL1 mRNA levels were evaluated by Northern blot analysis of total RNA from nd7 cells and from two clones transformed with pICL1b and displaying severe ICL-deficient phenotypes. The same blot, shown in Figure 4, was sequentially hybridized with the ICL1 probe (Figure 4a) and with the T1b probe (Figure 4b), the latter as control for the amount of RNA in each sample. When probed with ICL1, the RNA from uninjected control cells revealed only one band of the size expected for ICL1 mRNA (~0.85 kb). Very little ICL1 mRNA of this size was detected in the transformed clones; in both clones, however, a smear was visible with a stronger band at ~1.2 kb. The aberrantly sized RNA molecules were also found to hybridize weakly with a pUC18 probe, suggesting that they could represent transcripts from the microinjected plasmid DNA.

Microinjection of T1 or T4 TMP Coding Sequences Creates Secretory Mutants with Distinct Phenotypes

The TMP multigene family encodes proteins that coassemble at a specific stage of secretory granule biogenesis to form the trichocyst crystalline core. The resulting intracellular protein crystal is characterized by a highly constrained shape, which can be modified by recessive mutations that perturb intracellular trafficking and/or protein processing (Pollack, 1974; Adoutte *et al.*, 1984; Gautier *et al.*, 1994). The mutant trichocysts generally cannot attach to the cortical exocytotic sites and, therefore, cannot be secreted. Members of three TMP gene subfamilies, designated T1, T2, and T4, have been characterized. Although they share only 25% amino acid identity, T1, T2, and T4 proteins present a common organization and are predicted to have the same polypeptide fold (Gautier *et al.*, 1996). Within each subfamily, four to eight genes share ≥85% identity at the nucleotide level and code for very similar proteins (Madeddu *et al.*, 1995).

Plasmids bearing the coding sequences of TMP genes belonging to either of two different subfamilies (pT1b and pT4a, see Figure 1) were chosen for microinjection experiments. Wild-type cells were used as recipients, and the exocytotic activity of their clonal descendants was monitored by treat-

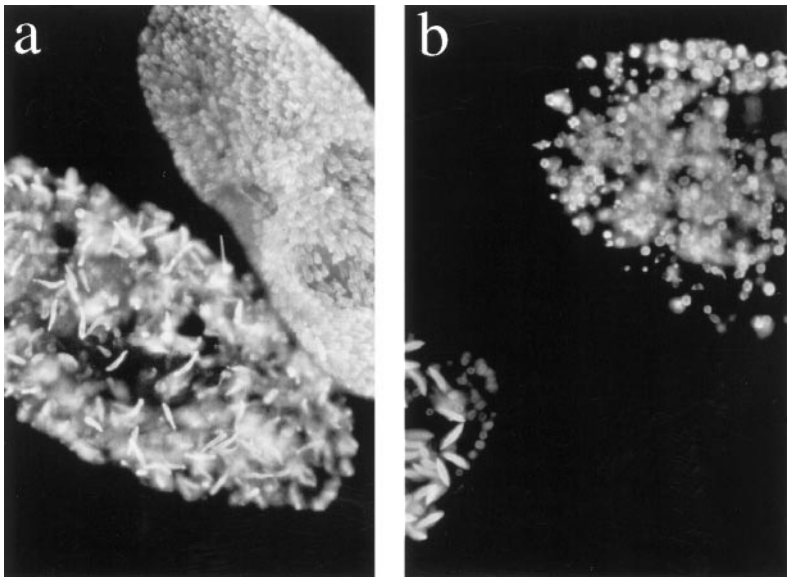

Figure 5. Phenotypic characterization of cells transformed with T1 and T4 coding sequences. Immunofluorescent images of trichocysts of a pT1b-transformed cell beneath an uninjected wild-type cell (a) and pT4a-transformed cells (20–25 divisions after microinjection) (b). Both pT1b- and pT4a-transformed cells presented undocked aberrantly shaped trichocysts, which tended to swell more readily than wild-type trichocysts under the fixation conditions used. Magnification, 500×.

ment with picric acid. All 11 cells microinjected with pT1b and 6 of 9 cells microinjected with pT4a gave rise to descendants that presented exocytosis-defective phenotypes. We selected 5 of these clones (3 transformed with pT1b and 2 transformed with pT4a) for further characterization. All pT1b transformants displayed a normal growth rate (four or five fissions/d), and pT4a-transformed cells displayed slow growth (three fissions/d).

The trichocysts of the transformed clones were examined in situ by immunofluorescent staining with an antibody that recognizes the entire set of TMP polypeptides, because TMP subfamily-specific antibodies are not yet available. As shown in Figure 5a, upper cell, wild-type cells have about one thousand trichocysts attached to cortical exocytotic sites, visualized as a dense layer of regularly shaped objects underlying the entire cell surface. Both pT1b (Figure 5a, lower cell) and pT4a-transformed cells (Figure 5b) presented undocked trichocysts free in the cytoplasm, consistent with loss of exocytotic capacity. The pT1b-transformed cells contained numerous trichocysts with subnormal morphology, reminiscent of the misshapen trichocysts of the mendelian *stubby* mutants, in which secretory granule biogenesis is perturbed (Pollack, 1974; Gautier *et al.*, 1994). The morphology of the trichocysts in pT4a-transformed cells was even more abnormal, consisting either of small nearly spherical shapes with irregular contours resembling flowers (Figure 5b, cell to the right) or of regular ellipses similar to grains of rice (Figure 5b, cell to the left). These forms were completely novel; pT4a trichocysts were unlike the trichocysts of any known mendelian trichocyst biogenesis mutant (Adoutte, 1988).

Analysis of TMP Subfamily mRNA Levels

Northern blot analysis of total RNA from cells of pT1b- and pT4a-transformed clones displaying severe mutant phenotypes revealed, as for pICL1 transformants (see Figure 4), reduced steady-state levels of mRNA of the expected size and some discrete bands corresponding to more slowly migrating species (Figure 6a). RNA of the secretory mutant tam8 (Beisson and Rossignol, 1975), whose morphologically normal trichocysts are unable to dock at the cortical exocytotic sites, is also shown in Figure 6. In tam8 cells, and also in wild-type cells depleted of their trichocysts by exocytosis, the steady-state level of TMP mRNAs is elevated by at least twofold with respect to the levels characteristic of resting wild-type cells (Galvani, Gautier, and Sperling, unpublished results). Steady-state TMP mRNA levels are thus inversely correlated with the state of occupation of the cortical trichocyst docking sites, suggesting that genes needed for trichocyst biogenesis are transcriptionally coregulated as a function of the number of trichocysts available for exocytosis at the cell surface. If we take into account this regulation, then the levels of T1 mRNA in pT1b-transformed cells and of T4 mRNA in pT4b-transformed cells is actually reduced to about 20% of the expected level, indicating that expression of most or all subfamily members is repressed.

Most significantly, pT1b- and pT4a-transformed cells presented high levels of the reciprocal steady-state mRNA (T1 mRNA in pT4a transformants and T4 mRNA in pT1b transformants), as expected for cells with undocked trichocysts. Thus, only T1 (or T4) gene expression was reduced in pT1b (or pT4a) transformants; expression of other TMP genes, belonging to

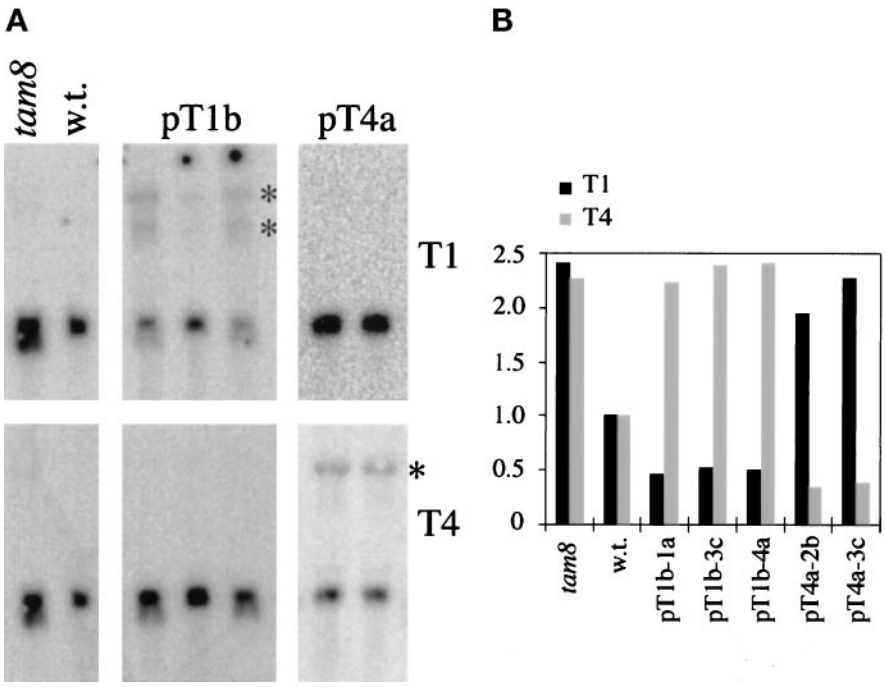

Figure 6. Northern blot analysis of pT1b and pT4a transformants. (a) Total RNA was prepared from tam8 and wild-type cells (left), three clones of pT1b-transformed cells (middle), and two clones of pT4a-transformed cells (right). The blots were sequentially hybridized with the T1-specific probe (top) and with the T4-specific probe (bottom). Asterisks denote aberrantly migrating species that hybridized with a pUC18 vector-specific probe. (b) Histogram of the steady-state levels of T1 and T4 mRNAs in the various clones was established by quantification of the Northern blots shown in a, using hybridization to an ICL1-specific probe to normalize for the amount of total RNA in each sample.

different subfamilies, was not reduced but rather physiologically up-regulated. Silencing can thus dissociate the expression patterns of functionally related genes that are normally coregulated.

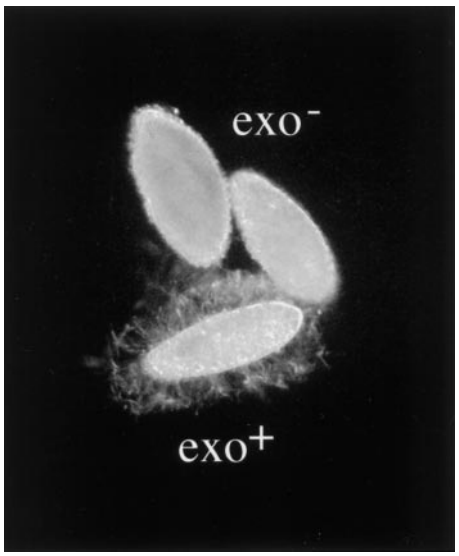

Figure 7. Evaluation of exocytotic activity of cells transformed with *ND7* sequences. The dark-field image shows cells treated with picric acid. Clonal descendants of cells injected with pND7−, which do not secrete any trichocysts (exo−), are shown above an nd7 cell whose exocytotic capacity has been restored (exo+) by transformation with a functional *ND7* gene (pND7+). The latter, which is indistinguishable from uninjected wild-type cells, is surrounded by a dense halo of trichocysts. Magnification, ×200.

Microinjection of the Coding Region of the Unique Copy Gene ND7 Generates Exocytosis-deficient Cells

The gene *ND7* was recently cloned by functional complementation of the mutation *nd7–1*, whose trichocysts are docked at the cortex but cannot undergo exocytotic membrane fusion (Skouri and Cohen, 1997). The previous genetic and the more recent molecular data show that *ND7* is unique in the *Paramecium* genome. To see whether silencing could be induced for genes that are not members of multigene families, wild-type cells were microinjected with plasmids containing the coding region of the *ND7* gene (Figure 1, pND7−). The exocytotic capacity of clonal descendants of 11 of 13 microinjected cells was found to be completely (Figure 7, upper cells) or partially impaired. Different clones of cells transformed with pND7− secreted, at most, a few trichocysts (0–30 per cell) when treated with picric acid, as opposed to ~1000 trichocysts for wild-type cells. Phase-contrast observations and immunofluorescence analysis confirmed that cells transformed with pND7− contained morphologically normal trichocysts, docked at their cortical exocytotic sites, like nd7–1 mutant cells.

Microinjection of a Functional ND7 Gene Does Not Lead to Silencing

An *ND7* gene construct containing the upstream promoter and downstream polyadenylation signals required for gene expression is able to rescue the exocytotic capacity of nd7–1 host cells when introduced into the macronucleus even at very low copy number

(Skouri and Cohen, 1997). Judging from our coinjection experiments above, fewer than 100 copies of linearized plasmid per macronucleus (i.e., <0.1 haploid genome equivalents) were found to be sufficient for complementation of the *nd7–1* mutation.

To see whether the same construct would continue to complement when introduced into the macronucleus at high copy number or on the contrary allow us to observe cosuppression, both wild-type and nd7–1 cells were microinjected with linearized pND7+ plasmid DNA (see Figure 1) at the same high concentration (10 mg/ml) used in the other microinjection experiments. The clonal descendants of all 19 wild-type cells microinjected with pND7+ continued to display a wild-type exocytotic phenotype, whereas the descendants of all 11 nd7 cells microinjected with pND7+ were rescued (Figure 7, lower cell), acquiring an exocytotic capacity indistinguishable from that of wild-type cells.

The amount of DNA in the clonal descendants of cells microinjected with pND7+ was analyzed by dot blot, to ensure that the plasmid was maintained at high copy number. Duplicate samples were prepared for 11 of the wild-type transformed clones and 6 of the nd7-transformed clones (see MATERIALS AND METHODS). The results are shown in Figure 8. For comparison, the number of copies of pND7− maintained in 15 clones of wild-type cells (see below) is also represented; copy number in these clones, expressed as haploid genome equivalents, varies from only a few copies to up to 200 copies. A similar distribution was observed after microinjection of pND7+ in wild-type cells. Most significantly, 5 of the wild-type clones maintain more than 40 copies of pND7+ per haploid genome, a value sufficient for complete *ND7* gene silencing with the pND7− construct. The six *nd7–1*-complemented clones maintain high copy number, between 50 and 100 haploid genome equivalents of pND7+. Thus, most of the pND7+-transformed clones have maintained the microinjected DNA at high copy number. We conclude that the silencing effect, at least for the *ND7* gene, is only obtained with constructs that lack flanking regulatory sequences.

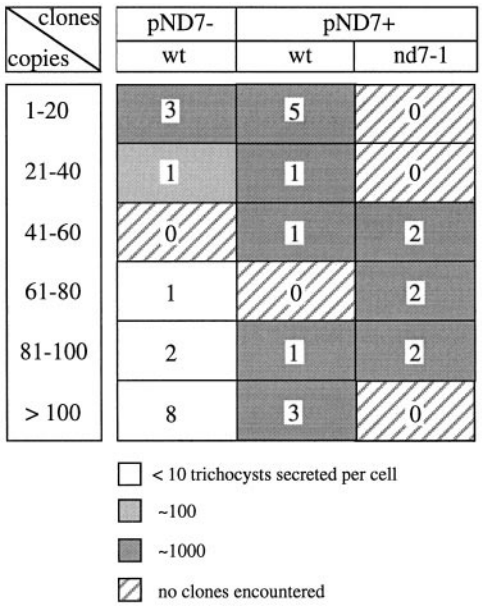

Figure 8. Evaluation of plasmid copy number in cells transformed with *ND7* sequences. The number of clones as a function of copy number (in haploid genome equivalents) of pND7+ maintained after microinjection of either wild-type or nd7 mutant cells is given. The phenotypes of the clones are also indicated (white background, fewer than 10 trichocysts secreted per cell; lightly shaded background, approximately 100 trichocysts; darkly shaded background, indistinguishable from wild-type control cells; hatched background, no clones encountered). These data are compared with the number of copies of pND7− maintained in clones of wild-type cells. The pND7− data are the same as in Figure 9. In all cases, copy number was determined by a dot blot 10 fissions after injection.

Quantitative Analysis of the Dose Dependence of ND7 Gene Silencing

In microinjection experiments carried out with genes from multigene families, in particular the ICL1 centrin genes, we were able to observe a range of mutant phenotypes, the ICL being either absent or more or less defective. The severity of the defects seemed to correlate with the amount of transforming DNA maintained in the clones, as judged by Southern blot analysis. We did not, however, attempt to evaluate this relationship quantitatively because the phenotypes most likely reflect reduced expression of not one cellular homologue but rather of several paralogous genes. In the absence of gene-specific probes (or antibodies), we cannot evaluate the degree to which the expression of each of the genes is affected or the precise phenotypic consequences.

The situation is quite different for *ND7*, because it is a unique copy gene and the phenotype resulting from reduced *ND7* expression can be evaluated quantitatively by counting the number of trichocysts secreted per cell in the presence of picric acid. We therefore carried out an experiment in which 15 wild-type cells were microinjected with pND7−; at 10 fissions postinjection, the phenotypes of the clones were evaluated by stimulation of exocytosis of a small population of cells with picric acid; and the copy number of the transforming DNA was quantified by dot blot of duplicate samples of 50 cells from each clone (Figure 9). The clones were also analyzed at later times, up to 20–25 fissions after injection, to verify that both copy number and phenotype were stable throughout vegetative growth. As shown by the scatter plot in Figure 9, *ND7* gene silencing has a threshold of approximately 25 haploid genome equivalents of pND7−. The existence of a threshold for silencing rather than a

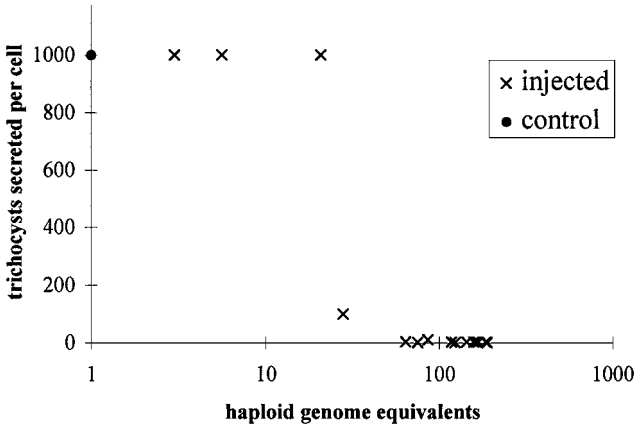

Figure 9. Quantitative analysis of plasmid-induced silencing. The average number of trichocysts secreted per cell is plotted as a function of the copy number of pND7− in the macronucleus. Exocytotic capacity was determined by treatment of 10–20 cells with picric acid. Although it is possible to count up to 50 secreted trichocysts per cell, higher values represent an appreciation of the density of the halo, compared with that of a wild-type cell assumed to secrete all of its ~1000 docked trichocysts. The copy number of plasmids, determined by a dot blot 10 fissions after injection, is expressed as the number of copies per haploid genome. ×, Each of the 15 transformed clones; ●, clone of uninjected control cells.

more graded response may be peculiar to the *ND7* gene, because very little gene product is required for exocytosis and given the all-or-none nature of membrane fusion events.

DISCUSSION

We report herein that delivery of cloned gene fragments to the *Paramecium* macronucleus by microinjection at high copy number can lead to reduced expression of the homologous cellular genes. The injected DNA corresponded to transcribed sequences, lacking any flanking regulatory sequences, of the single-copy gene *ND7*, which is required for exocytotic membrane fusion (Skouri and Cohen, 1997), and of members of the ICL and the TMP multigene families, whose products coassemble to form, respectively, the ICL and the trichocyst crystalline matrix (Madeddu *et al.*, 1995, 1996). Our experimental protocol gave rise to transformants that presented specific defects in the subcellular structures containing the products of the target genes. For the single-copy gene *ND7*, we were able to obtain a phenotype indistinguishable from that conferred by the *nd7–1* hypomorph allele. In experiments involving members of the two multigene families, molecular analysis of the transformed cells showed that gene silencing probably affects all paralogous genes presenting a high degree of sequence identity to the microinjected DNA.

These observations are significant for three reasons. First, given the similarities to transgene-induced silencing in plants and fungi, the observations could represent a related phenomenon in a new phylum, the ciliates. Secondly, by allowing targeted reduction in gene expression, the phenomenon described herein provides a tool for reverse genetics. Thus, with recently developed methods for cloning genes by functional complementation (Haynes *et al.*, 1996; Skouri and Cohen, 1997), the possibility of inactivating specific genes establishes *Paramecium* as a powerful model system. Lastly, gene silencing provides an ideal means of analyzing the significance of the multigene families that *Paramecium* uses to encode proteins that coassemble to build up complex subcellular structures. This study presents the first experimental evidence that the members of these multigene families are not functionally redundant.

Gene Silencing in Paramecium

As for silencing phenomena described in other systems, mainly transgenic plants (for reviews Flavell, 1994; Matzke and Matzke, 1995; Baulcombe, 1996; Bingham, 1997; Depicker and Van Montagu, 1997; Stam *et al.*, 1997), the effects we observed appear to be both dose dependent and homology dependent. Dose dependence was established quantitatively for the single-copy gene *ND7*, which presented a threshold for silencing of ~25 copies of exogenous DNA per haploid genome. Moreover, in the experiments with multigene families, where the implicated cellular structures could be visualized by immunofluorescent staining, a range of defects were observed, and their severity seemed to correlate with the quantity of transforming DNA maintained in the different clones, as judged by Southern blot hybridization.

Homology dependence is consistent with our observations that silencing only affected genes containing sequences closely related to the sequence of the transforming DNA. With the exception of *ND7*, the genes that were investigated belong to multigene families, organized in subfamilies of genes that share ≥85% nucleotide identity. Homology dependence was most clearly demonstrated by experiments involving genes of two different TMP subfamilies, T1 and T4. Because the exon probes used for mRNA analysis were subfamily-specific (only the more divergent intron sequences provide gene-specific markers; Madeddu *et al.*, 1995, 1996), we cannot say whether expression of some of the genes in a subfamily is completely abolished and expression of others is unaffected or whether the expression of all genes in a subfamily is reduced. Nonetheless, steady-state mRNA levels are clearly reduced only for genes belonging to the same subfamily as the transforming DNA, whereas expression of members of the other subfamily is not directly affected.

Other features of gene silencing in *Paramecium* suggest that the mechanism is posttranscriptional. It is now clear that the reduction in steady-state mRNA characteristic of gene silencing in transgenic plants and fungi can result either from mechanisms that operate at the transcriptional level via DNA–DNA pairing and DNA methylation, most clearly demonstrated for RIP and MIP in fungi (for review Selker, 1997), or from posttranscriptional events affecting RNA stability (for reviews Baulcombe, 1996; Depicker and Van Montagu, 1997; Stam *et al.*, 1997). The fact that microinjection of constructs without regulatory sequences appears to be both necessary and sufficient to generate silencing in *Paramecium* speaks for a posttranscriptional mechanism: transcriptional silencing in plants needs homology in the promoter region, whereas posttranscriptional silencing in plants and quelling in *Neurospora* only require homology of transcribed sequences (Cogoni *et al.*, 1996; Jorgensen *et al.*, 1996; Park *et al.* 1996; Thierry and Vaucheret, 1996). Moreover, for quelling, deletion analysis indicates that the constructs that lack promoters are the most efficient (Cogoni *et al.*, 1996). Nonproductive RNA molecules, related to the increased mRNA turnover thought to be responsible for posttranscriptional gene silencing have been identified in some systems (Cogoni *et al.*, 1996; Metzlaff *et al.*, 1997). Likewise, we have detected aberrantly sized RNA molecules in transformed clones that probably represent transcription products from the microinjected DNA. Molecular characterization of these abnormal RNA species is needed to establish their nature and eventual role.

The remarkable feature of the silencing phenomenon described herein is the extremely high efficiency (up to 100%) with which specific mutant phenotypes can be generated. On the basis of the data obtained so far, silencing appears to offer a convenient means of inactivating any *Paramecium* gene. Moreover, unlike gene disruption by homologous recombination, available in several model systems including the ciliate *Tetrahymena* (Gaertig *et al.*, 1994), silencing is not an all or none phenomenon. It is possible to obtain partial phenotypic effects, and this should prove particularly useful for functional analysis of essential genes.

It has been reported that the frequency and the strength of gene silencing are determined by the repetitiveness and organization of the transgene, the effects being greatest when multiple copies are inserted in the host genome in a repeat configuration (Depicker and Van Montagu, 1997 and references therein). In *Paramecium*, the microinjected DNA is not integrated in the macronuclear chromosomes but is maintained in an episomal state, as a series of linear multimeric molecules. The high efficiency with which we observed silencing may thus be a consequence of the way in which foreign DNA is processed and maintained in a repeat configuration in these autonomously replicating pseudochromosomes, which seems to be peculiar to *Paramecium* (Bourgain and Katinka, 1991; Kim *et al.*, 1992). This organization may also explain the requirement for high transgene copy number in our experiments. If only tandem repeats of the transgene were effective in silencing, then the effective copy number would be much lower than the 25 haploid genome equivalents we measured, because most of the copies are present as "monomers" (see Figure 2). Further investigation of the requirements for silencing of different *Paramecium* genes, in terms of the dosage, the nature of the injected sequences, and their organization in the macronuclear pseudochromosomes, should help clarify the molecular basis of the process and its relationship to gene silencing in other organisms.

Multigene Families and Morphogenesis

The discovery that different subcellular structures in *Paramecium* are built up from heterogeneous mixtures of related polypeptides (Tindall *et al.*, 1989; Garreau de Loubresse *et al.*, 1991; Sperling *et al.*, 1991; Coffe *et al.*, 1996) encoded by families of coexpressed genes (Madeddu *et al.*, 1995, 1996) raises the question of the selective pressure that has driven the generation and maintenance of these unusual gene families in a unicellular organism. One hypothesis is that these families arose through gene duplications in response to a need for large amounts of protein, that the gene products are interchangeable in the structures they form, and that the members of the gene families have accumulated neutral mutations compatible with the molecular design needed for assembly. A more attractive hypothesis stipulates that the heterogeneity of structural proteins assured by the multigene families is necessary for the edification of geometrically complex dynamic structures and predicts that the different genes are not redundant but rather have distinct functions in organelle assembly.

The data presented herein clearly speak in favor of the latter hypothesis. Reduced expression of genes belonging to specific ICL or TMP subfamilies compromises assembly of an intact ICL or a functional trichocyst. Because silencing appears to affect all closely related paralogues, it remains possible that genes within a subfamily are at least partially redundant. However, depending on which TMP subfamily was affected, the phenotypes of the mutant trichocysts obtained were different, providing a strong argument that the effects of silencing on trichocyst morphogenesis result from qualitative rather than quantitative changes in the pool of TMPs available for assembly. Further biochemical, morphological, and structural studies of the defective organelles that we can now create by silencing *Paramecium* genes should help understand the morphogenesis of *Paramecium* secretory

granules and cortical cytoskeletal arrays, as well as the logic behind the use of multigene families to build them.

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