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Introducing Antisense Oligodeoxynucleotides into *Paramecium* via Electroporation

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ABSTRACT. A method utilizing electroporation to deliver antisense oligodeoxynucleotides into *Paramecium tetraurelia* has been developed. For these studies antisense oligonucleotides directed to different regions of the calmodulin mRNA were used. It was found that a pulse delivered at 150–250 V (375–625 V/cm field strength) for 3.9–4.2 ms using a 275 μF capacitor with resistance set at 13 Ohms was sufficient to achieve measurable incorporation of fluorescently-labeled oligodeoxynucleotides in up to 95% of the cells treated. Optimal parameters included using oligodeoxynucleotides of at least 12 bases in length with a 3′ blocking group at a dose of around 10 μM. In addition, multiple oligodeoxynucleotides directed to the same target mRNA resulted in at least a 10-fold reduction in the dose of oligodeoxynucleotide required to achieve the desired effects. Taken together, these results indicate that the use of antisense oligodeoxynucleotides can be an easy and useful method for linking genes to specific functions in *Paramecium tetraurelia*. Finally, this report discusses how different 3′ blocking groups and the use of combinations of oligodeoxynucleotides directed to different regions of the same target mRNA can help address concerns about specificity.

Supplementary key words. Calcium-dependent K^+ channel, calcium-dependent Na^+ channel, calmodulin, cholesterol-linked oligodeoxynucleotides, hexanol-linked oligodeoxynucleotides, protozoan behavior.

ANTISENSE oligodeoxynucleotides (ODN) can be used to transiently down-regulate a gene's expression and thereby help identify the role of the gene product in the cell [2]. Their ease of use makes antisense ODN an attractive method for utilizing a "reverse genetics" approach for the assignment of function to cloned genes. Previously, we described the microinjection of antisense ODN as a viable methodology in the ciliated protozoan, *Paramecium tetraurelia* [5]. In that report, antisense ODN were targeted against calmodulin mRNA resulting in abbreviated behavioral responses that mimicked the type of behavioral responses seen in cells with a mutant calmodulin gene that does not properly activate the calcium-dependent Na^+ current. Thus, it was concluded that the antisense ODN reduced the levels of calmodulin sufficient to affect the activation of the calcium-dependent Na^+ current.

Recently it was reported that electroporation can be used as a method for incorporating ODN into cells [1] and, in an effort to improve the ease of use of this technology in ciliates, we have developed a procedure for introducing antisense ODN by electroporation. Calmodulin was again chosen as the target because of its well-characterized role in the control of behavior in *Paramecium* [6, 14]. In addition, we have characterized some of the parameters important for the use of this methodology in *Paramecium*.

MATERIALS AND METHODS

Cell cultures and media. The wildtype strain 51S of *Paramecium tetraurelia* and a calmodulin mutant strain that is derived from 51S and lacks a calcium-dependent K^+ current (*cam1*) were used in these studies. The cells were grown in either axenic medium [17] or in wheat grass medium [8] that was inoculated with *Klebsiella pneumoniae,* as required. Exhausted wheatgrass medium was prepared by removing all *Paramecium* cells from a stationary culture by centrifugation and filtration techniques and autoclaving as needed.

Oligonucleotide synthesis. Sequences and structures of the oligonucleotides (ODN) used are indicated in Table 1. Various conjugate groups were added to the 3′ or 5′ end of the ODN. ODNs were prepared on an Applied Biosystems Model 394 synthesizer (Foster City, CA) using the 1 μmole synthesis protocols supplied by the manufacturer. Protected β-cyanoethyl phosphoramidites, controlled pore glass (CPG) supports, deblocking solutions, cap reagents, oxidizing solutions, and tetrazole solutions were purchased from Glen Research (Sterling, VA). The 3′-hexanol (J) and cholesterol (H) modifications were introduced into ODNs through the use of the appropriate modified CPG support. Chemical syntheses of these modified CPGs and properties of the 3′-modified ODN have been described in detail [4]. Hexanol was directly bonded to the 3′-phosphate, whereas the cholesterol modifications were attached via a hydroxyprolinol linker. The 5′-fluorescein modification (F) was introduced into PAR001JF on the DNA synthesizer using FAM phosphoramidite (Applied Biosystems). Preparative HPLC purification, detritylation, and butanol precipitation of the synthetic ODN was carried out as previously described [4]. To ensure complete removal of the triethylammonium acetate HPLC buffer, each ODN was taken to dryness with 3 equivalents (with respect to the nucleotides) of sodium bicarbonate. After reconstituting with 1 ml of water, the ODN concentration was determined from the UV absorbance at 260 nm in phosphate-buffered saline, pH 7.2. An extinction coefficient was calculated for each ODN, correcting for the molecular weight of appended modifications. All modified ODNs were analyzed by reverse phase HPLC as previously described [4]. ODN purity was further confirmed by denaturing polyacrylamide gel electrophoresis (PAGE). The nucleotide bands were visualized by silver staining. All modified ODNs were greater than 95% pure by HPLC and yielded one major band by PAGE.

Delivery of oligonucleotides by cell electroporation. Approximately 200 ml of a late-log to early-stationary phase cell culture were harvested by centrifugation in a clinical centrifuge (500 *g*). The cell pellet (0.25–0.5 ml packed cell volume) was washed twice in 100 ml of 10 mM HEPES, pH 7.2. The pellet was then resuspended in 5 volumes of 10 mM HEPES, pH 7.2. A 250 μl aliquot of the cell resuspension (at approximately 10^5 to 10^6 cells per ml) was then placed in an electroporation cuvette (0.4 cm gap). The cell suspension was mixed in the cuvette with the ODN at varying concentrations and electroporated using an Electrocell Manipulator 600 (BTX Inc., San Diego, CA). Resistance was set at 13 Ohms. A pulse was delivered at 150–250 V (field strength 375–625 V/cm) for 3.9–4.2

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Table 1. Oligodeoxynucleotides (ODN) used in electroporation of *Paramecium* cells.

ODN[a]	Modifications	Sequence (5′ → 3′)	Features/Location
ODNs of Standard Length (24-mer)			
PAR001	J, H, JF	TAATTATTCAGCCATTTATTAGTT	AS to 5′ end
PAR002	J, H	TACTATATCATGGATCATAATTAA	Random Control
PAR004	J	TTCAATTAATTCTTCCTCAGAATC	AS to internal site
PAR501	J, H	AACTAATAAATGGCTGAATAATTA	Sense Control
PAR017	J, H	TACTATCTGTTAGTATATTACTTA	Scrambled PAR001
ODNs of Varying Length (6-mer to 36-mer)			
PAR013	J, H	TTCTGTTAATTATTCAGCCATTTATTAGTTGATTTA	36-mer AS to 5′ end
PAR012	J, H	TTCAGCCATTTA	12-mer AS to 5′ end
PAR014	J, H	GCCATTTATT	10-mer AS to 5′ end
PAR015	J, H	CCATTTAT	8-mer AS to 5′ end
PAR016	J, H	CATTTA	6-mer AS to 5′ end

[a] The standard nomenclature for the various ODN sequences used is the abbreviation PAR (for *Paramecium*) followed by a three digit number. This is followed by a letter that refers to the type of modification found on the ODN. (J) = hexanol on 3′ end; (H) = cholesterol on 3′ end; (JF) = hexanol on 3′ end and fluoroscein on the 5′ end. AS = antisense.

ms using a 275 μF capacitor. The exact field strength used varied with each experiment and was determined empirically by identifying the field strength that resulted in approximately 50–60% cell mortality. All electroporations were performed at room temperature. After electroporation, cells were placed into 64 volumes of 10% exhausted wheat grass (v/v) in Resting Solution (1mM HEPES pH 7.2, 1mM $CaCl_2$, 1mM KCl) and allowed to incubate overnight at 28° C. When using a GIBCO BRL Cell Porator Electroporation system, conditions were altered slightly. Cells were prepared essentially as described above for the BTX Electrocell Manipulator. However, after the final resuspension in 10mM HEPES cells were electroporated at a field strength of 2,000V/cm, 300 μF using the low resistance setting. After electroporation, the cells were washed three times in citric acid resting solution (1mM $Ca(OH)_2$, 1mM citric acid, 1mM KCl, 1mM Tris base, pH 7.2) and incubated overnight at 22° C.

Measurement of fluorescent ODN uptake following electroporation. A specially prepared calmodulin antisense ODN (PAR001JF) containing a fluorescein molecule covalently attached to the 5′ end was used to measure the percentage of cells that took up the ODN following electroporation. Following the electroporation procedure, cells were allowed to rest for 1 h. They were then washed twice in 10% exhausted wheatgrass in Resting Solution and monitored for uptake of the fluorescent molecule by two different methods. 1) Individual cells were selected at random and observed using a Zeiss inverted-epifluorescence microscope at 400×. Cells were scored for their relative amount of fluorescence as compared to controls. 2) A Fluorescence-Activated Cell Scanner bench top analyzer with a dedicated HP340 computer running the LYSIS II data acquisition and analysis software was utilized to determine the number of cells that took up the labeled ODN. There was no detectable surface binding of the ODN in control cells as verified by epifluorescence microscopy.

Testing of cell swimming behavior. Cells were tested in two different ionic solutions in order to observe their swimming responses [7, 15]. The Na/TEA test solution was 10 mM NaCl and 5 mM TEA in Resting Solution. All cells were first placed in Resting Solution for approximately 5–10 min before transferring them to the test solution. The Na/TEA solution allowed us to monitor for inhibitory effects on the calcium-dependent Na^+ channel (decreased backward swimming relative to controls) and the calcium-dependent K^+ channel (increased backward swimming relative to controls). The duration of backward swimming time for individual cells was monitored and timed under a dissecting microscope using a stopwatch. All experiments used between 12–33 cells per treatment. Control cells received either a sense ODN (PAR501J or PAR501H) or a scrambled antisense-sequence ODN (PAR002J). These gave identical results and were used interchangeably. Effects upon the voltage-dependent Ca^{2+} channel were monitored by testing the cells in Resting Solution containing 30 mM KCl. The Mann-Whitney *U*-test was used for all behavioral data described in this paper.

Generation of anti-*Paramecium* calmodulin polyclonal antibody. The *Paramecium* calmodulin gene was cloned into the plasmid pET-11D (Novagen, Madison, WI) and expressed in *E. coli* strain JM109(DE3) as previously described [19]. This construct puts the *Paramecium* calmodulin gene (with all TAA and TAG codons changed to CAA and CAG codons, [10]) behind the IPTG inducible *Lac Z* promoter. Twenty ml of an overnight culture of transformed cells were added to 1 liter of LB/Ampicillin (100 μg/ml) and shaken 2 h at 37° C until the absorbance at 550 nm was about 0.5. The inducer, IPTG, was then added to a final concentration of 1 mM and the culture shaken at 37° C for 3 h. Bacteria were collected by centrifugation at 2,000 *g* for 15 min and bacterial pellets washed twice with 40 mM Tris buffer, pH 7.5. Pellets were then resuspended in 10 ml of a 2.4 M sucrose solution with 100 μM EDTA and incubated on ice for 30 min. Cells were lysed with 100 μg/ml lysozyme and boiled 5 min to eliminate unwanted proteins. After centrifugation at 30,000 *g* for 2 h at 4° C, $CaCl_2$ was added to the supernatant to a final concentration of 5 mM and the solution applied to a phenyl sepharose CL-4B (Sigma, St. Louis, MO) column. After washing with 50 ml of a 50 mM Tris HCl, 0.5 mM NaCl solution, the enriched, expressed *Paramecium* calmodulin was eluted with 5 ml of 50 mM Tris HCl and 1 mM EGTA solution. Calmodulin was concentrated with Centriprep-10 mini-concentrators to a concentration of 2 mg/ml.

Polyclonal antibody was generated by injection of rabbits with calmodulin/Complete Freund's Adjuvant suspension (0.5 mg calmodulin subcutaneous, 0.5 mg calmodulin intradermal in 2 wk intervals four times) by Lampire Biological Laboratories. Antibody specificity was assessed by Western blot of the bacterial expression product and of the *Paramecium* cell extract (as prepared for ELISA).

ELISA quantification of *Paramecium* calmodulin levels. *Paramecium* electroporated with either sense or antisense calmodulin oligodeoxynucleotide were screened for behavioral responses that were indicative of successful calmodulin reduction. For wildtype cells this was a backwards swimming response of

Table. 2. Comparison of methods to deliver oligodeoxynucleotides (ODN) into *Paramecium* cells assayed using backward-swimming times.

Cell type (treatment)	ODN treatment[1]	Average backward swimming time (in sec.)[2]	Range (in sec.)
Wildtype Microinjected	Control	16.0 ± 2.4	12 to 18
	Antisense	1.3 ± 0.2	0 to 4
Wildtype Electroporated	Control	14.4 ± 2.6	8 to 19
	Antisense	5.4 ± 4.9	0 to 17
Cam-1 Microinjected	Control	102.4 ± 9.6	81 to 122
	Antisense	51.6 ± 11.1	39 to 73
Cam-1 Electroporated	Control	87.8 ± 15.2	60 to 110
	Antisense	48.5 ± 6.7	38 to 61

[1] All antisense ODN treatments were with PAR001J. Control ODN treatments were with PAR501J or PAR002J (controls) (Table 1).

[2] Mean of backward swimming times ± standard deviation. 12–24 cells per experiment.

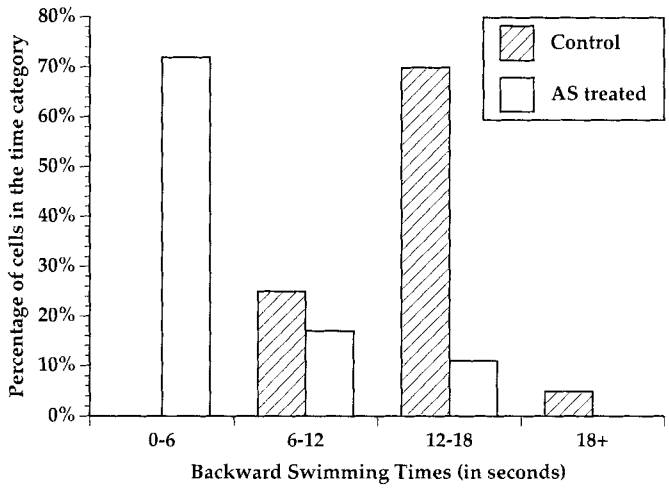

Fig. 1. Distribution of backward swimming times of *Paramecium* after calmodulin AS treatment. Wildtype cells (51S) were electroporated in the presence of 10 μM PAR001J (Table 1) and incubated in 10% exhausted wheatgrass in resting solution at 28° C for 9 h before testing for backward swimming responses in Na/TEA testing solution. The cells are grouped according to the length of time (in seconds) that they swam backwards in this testing solution. The hatched columns represent cells electroporated with a control oligo-deoxynucleotide (ODN). The dotted columns represent the antisense (AS)-treated cells. Note that 26% of the AS ODN-treated cells maintained backward swimming longer than 6 s.

less than 5 s. Thirty cells of each treatment type were suspended in 200 μl of 1 mM KCl, 1mM $Ca(OH)_2$, 1 mM citric acid buffer, pH. 7.0, and sonicated 1 min on ice. The preparation was then boiled 5 min and cooled on ice for 5 min before centrifugation at 12,000 *g* for 15 min. Fifty μl aliquots of each sample, along with 50 μl aliquots of expressed *Paramecium* calmodulin serial dilutions were then applied to wells of an ELISA plate and allowed to sit overnight at room temperature. The following day, plates were blocked for 1.5 h with phosphate buffered saline (PBS) with 0.05% Tween and 0.25% bovine serum albumin. Fifty μl of the rabbit anti-*Paramecium* calmodulin polyclonal antibody were then applied in triplicate to the plate at a 1:5,000 dilution with PBS/Tween. Pre-immune sera were applied to the fourth aliquot of each sample as a baseline negative control, also diluted 1:5,000 with PBS/Tween. After a 2-h incubation, plates were washed 3 times with PBS/Tween and 50 ml of a secondary goat anti-rabbit alkaline phosphatase conjugate (Sigma) were added to the plate at a dilution of 1:5,000. Plates were washed with PBS/Tween 3 times after 2 h of incubation and developed with 1 mg/ml of p-nitrophenol substrate in a 10% diethanolamine, 1 mM $MgCl_2$ buffer, pH 9.5, for 40 min. Optical densities at 405 nm were obtained from an automatic ELISA plate reader (Bio-Tek). Optical densities were converted to pmoles calmodulin for comparison purposes by referring to a standard curve run in parallel on each plate using known amounts of the expressed *Paramecium* calmodulin.

RESULTS

Optimizing conditions for delivery of antisense oligodeoxynucleotides via electroporation. Cells that had undergone electroporation in the presence of either the antisense or control oligodeoxynucleotide (Table 1) were tested for a reduction in backward swimming response in the Na/TEA testing solution. Previously, wildtype cells microinjected with PAR001J had exhibited a marked reduction in their swimming response in the Na/TEA test solution, indicative of a reduction in the calcium-dependent Na^+ current [5, Table 2]. The cells electroporated with the PAR001J ODN also had reductions in their backward swimming times in the Na/TEA testing solution (Table 2). The greatest reductions in backward swimming times were typically observed 10–12 h after electroporation and measurable effects would last for an additional 4–8 h at 28° C. Both wildtype and the mutant strain *cam1* were compared for the use of electroporation and microinjection as methods for the introduction of antisense ODN into cells (Table 2). Both types of cells electroporated with the calmodulin antisense ODN gave reduced backward swimming responses relative to both types of control AS treated cells (Table 2). Cells grown under axenic or wheatgrass conditions were equally responsive to treatment with only minor differences (data not shown).

During the course of the initial experiments, the electroporated wildtype cells as a group showed more variability in their behavioral responses after treatment with the antisense ODN when compared with the microinjection experiments (Table 2). One explanation for this increased variability is that electroporation may not result in the uniform incorporation of the ODN among the population of cells compared to microinjection (in which the uniform incorporation of ODN can be monitored visually). In order to examine this possibility, electroporation of the fluorescently-labeled ODN, PAR001FJ, was performed and the amount of fluorescence incorporated was determined. Visual examination by epifluorescence microscopy revealed that individual cells received different amounts of the fluorescent ODN. FACS analysis indicated that not all viable cells received measurable amounts of the ODN when compared to the background fluorescence of control cells: the percentage of cells receiving measurable amounts of fluorescent dye generally increased with increasing field strength and usually plateaued between 80–95%. This increase was accompanied by a decrease in the percentage of viable cells after treatment (Fig. 2). The percentage of cells that apparently did not incorporate significant amounts of the labeled ODN (5–20%, as determined by epifluorescence microscopy and FACS analysis) correlated well with the percentage of cells (8–29%) that did not appear to be affected by the antisense treatment throughout the 24-h time course of the experiment (see Fig. 1 for an example at one timepoint). These ranges for successful electropermeabilization of cells have been described before [13]. However, a previous report introducing antisense ODN specifically by electroporation has described nearly 100% incorporation into surviving

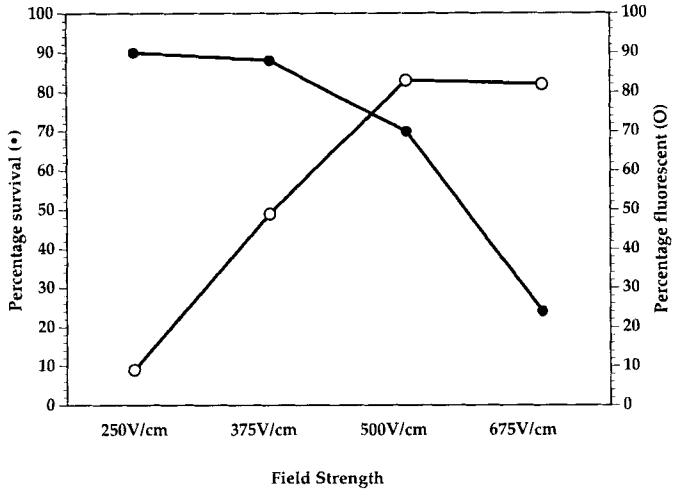

Fig. 2. Field strength versus relative survival of *Paramecium* (percentage survival) and relative uptake of oligodeoxynucleotide (ODA) (percentage fluorescent). Cells were electroporated in the presence of 10 μM PAR001FJ (Table 1) and washed twice in resting solution. Fluorescence uptake (○) was examined using a FACS bench top analyzer or individually using an epifluorescence equipped microscope. Cell survivability (●) was determined by counting viable cells before and after electroporation. The averages of 3 experiments (% Fluorescence) and 2 experiments (% Survivors) are shown.

cultured mammalian cells [1]. The reason for the difference is unknown but may be due to differences in cell type or experimental criteria for scoring cells as to whether they received the labeled ODN.

Electroporation conditions were optimized by examining the effects of growth phase and various electrical parameters upon the effectiveness of AS ODN incorporation (using PAR001FJ) and behavioral effect (using PAR001J). The most reproducible behavioral data were obtained when late-log to early-stationary phase cells were used. Cells from early-log or late-stationary growth phases gave more variable results and were less predictable as to the timing of the observed behavioral effects. The most effective electroporation parameters were a field strength in the range of 375–625 V/cm, a resistance set at 13 Ohms, and 275 μF capacitance using a BTX Electrocell Manipulator 600 (BTX Inc., San Diego, CA). This combination generated a pulse of 3.8–4.1 ms in duration. During an actual experiment, the field strength that resulted in approximately 50–60% cell death was used.

The effects of dose, ODN size, and various 3′ modifications upon AS ODN efficiency. In an effort to improve the sensitivity of the electroporation procedure, several additional parameters were tested. For the purpose of these experiments, *cam1* mutant cells were used to simplify our analysis. This was because we have observed that sufficiently high doses of antisense ODN can apparently reduce the calmodulin levels in wildtype cells sufficiently to have effects upon both the Ca^{2+}-dependent K^+ and Na^+ currents (Yano et al., unpubl. data). Reducing the Ca^{2+}-dependent Na^+ current in wildtype cells will reduce the behavioral response, while reducing the dependent K^+ current will increase the response [9]. Since the *cam1* mutant cells do not have a Ca^{2+}-dependent K^+ current, the only behavioral effect possible in calmodulin AS ODN-treated *cam1* cells is a reduction in backward swimming (due to a reduction in the activity of the Ca^{2+}-dependent Na^+ current). Thus, the potential complications of having inhibitory effects upon two opposite-acting currents is eliminated, allowing for a less complicated analysis of the various parameters tested. In general, when *Paramecium* lacks the Ca^{2+}-dependent K^+ current, they swim backwards in a Na/TEA Testing solution for about 90–120 s. Previously we had shown that *cam1* cells microinjected with calmodulin AS ODN at a concentration of 1.6–3.2 μM will exhibit a 50% reduction in their backward swimming response in a Na/TEA testing solution [5]. There was never any observation of antisense-treated *cam1* cells that swam backwards for significantly longer than control cells.

A dose-response curve of the ODN in the electroporation procedure indicated that the maximum effect upon the behavioral response of *cam1* cells in Na/TEA plateaus at about 10 μM (Fig. 3). A higher concentration of antisense ODN (at 20 μM) was not significantly different than that seen at 10 μM. A concentration of 10 μM was higher but still comparable to the concentration (1.6–3.2 μM) that was effective in the microinjection experiments [5]. The difference seen may be due to less efficient introduction of ODN into the cell cytoplasm via electroporation. For the remainder of the experiments described below (except where noted), a single ODN concentration of 10 μM in the electroporation cuvette was used because it was the minimally effective dose needed to achieve the maximum effect upon *cam1* cells and was therefore not as likely to generate nonspecific effects in subsequent experiments.

A recent report that indicated that the simultaneous use of multiple ODN directed to the same target gene acted synergistically, thus reducing the dose necessary to realize a specific AS effect [12]. This synergy resulted in a 10-fold reduction of the total amount of ODN that was necessary for an effective treatment; seeing such a synergistic effect can be taken as evidence that a treatment is specifically affecting the target mRNA [12]. To test for synergistic effects in *Paramecium*, two ODN, PAR001J and PAR004J, were combined in the same electrocuvette. The PAR001J was directed to the 5′ end of the calmodulin gene while the PAR004J was directed to an internal target sequence. Each individual ODN significantly decreased the behavioral response when electroporated at 10 μM but not at 1 μM (Fig. 3); while a 1 μM dose is ineffective when only one ODN is used, when both are combined (at 1 μM combined total, or 0.5 μM each) there is a significant effect upon the behavioral responses of the cells (Fig. 3). Thus, a synergistic effect can be realized in *Paramecium* as well.

The minimal effective length of AS ODN was determined using various sequences designed to hybridize close to the initiation codon (Table 1). The minimal length that was effective at 10 μM was found to be 12 bases (Fig. 4), which is similar to what is seen in other systems [3, 18, 16]. A 10-base ODN was not effective at this concentration, while an ODN of 36 bases was as effective as the 12-base ODN (at 10 μM) with no evidence of nonspecific effects at this dose (Fig. 4).

Earlier work on *Paramecium* indicated the necessity of using a hexanol group on the 3′ end of the ODN for biological effectiveness [5]. Improved antisense potency of 3′-modified ODN is presumably due to resistance to nuclease degradation in cells. The 3′ hexanol and cholesterol modifications have been shown to have the same serum stability when cultured with hepatoma cells [4]. The cholesterol 3′ modification was tested with PAR001 in order to compare its effectiveness at 10 μM. Our results indicated that it results in an equally effective ODN at a dose of 10 μM (see Fig. 4).

The electroporation of antisense ODNs reduces calmodulin protein. To further ensure that our antisense treatment was having specific effects upon calmodulin protein levels an ELISA was developed using an antibody against bacterially-expressed *Paramecium* calmodulin. The ELISA was sensitive enough to detect reliably, differences in calmodulin using only

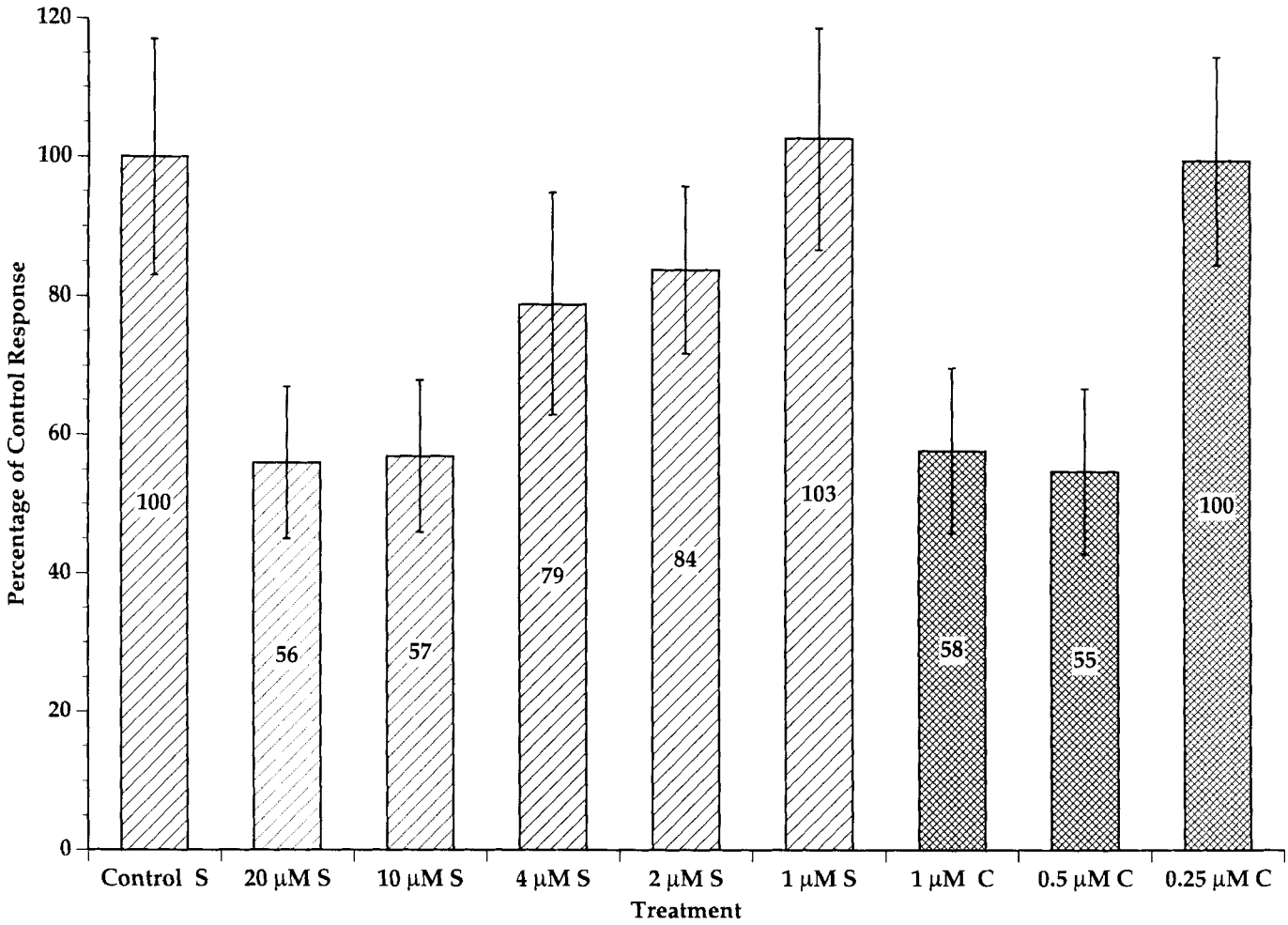

Fig. 3. Dose response of *Paramecium* to calmodulin antisense (AS) oligodeoxynucleotides (ODN). *Cam1* cells were electroporated in the presence of various concentrations of the AS ODNs, PAR001J and PAR004J. Treatments noted with an S and the control used PAR001J (Table 1) alone at the indicated dose (1–20 μM). PAR004J (Table 1) gave equivalent dose response curves (data not shown). Treatments noted with a C used a *combined* dose (0.25 μM, 0.5 μM or 1 μM) of both PAR001J and PAR004J. After electroporation all cells were incubated in 10% exhausted wheatgrass in resting solution at 28° C for 8–12 h and tested in Na/TEA testing solution at 1-h intervals. Each experiment was performed four times and the values presented are from a typical experiment. Control cells swam backwards on average between 80–120 s under the conditions used. All values were normalized to the backward-swimming response of the control treated cells tested at the same time. A normalized value of 100% was used for the control cells with a standard deviation given for all treatments to reflect the amount of variability seen in a typical experiment. The effects seen at 10 μM and greater concentrations were significantly different than the control values ($p < 0.001$). The differences at 10 μM and 20 μM were not significantly different from each other. The effects seen using combined ODNs at doses of 1 μM, (1 μM C) and 0.5 μM (0.5 μM C) were significantly different than the control ($p < 0.001$). The effects seen with a combined dose of 0.25 μM (0.25 μM C) were not significantly different than the controls ($p < 0.001$). The Mann-Whitney *U* Test was used for all statistical tests.

20–40 cells. For these experiments, antisense-treated wildtype cells that gave short backward swimming responses (<5 s) were pooled and compared to a randomly selected population of control ODN-treated cells.

Based on three experiments, the average decrease in calmodulin protein of cells treated with calmodulin antisense ODNs compared to cells treated with calmodulin sense ODNs was 32.3 ± 13.1%. Due to the small number of cells examined for each measurement and the harsh treatment used to partially purify calmodulin protein, data were normalized by comparing total calmodulin protein isolated from equal numbers of similar-sized cells. Thus, calmodulin levels from approximately ten antisense ODN-treated cells were compared to calmodulin levels from approximately ten similar-sized, control ODN-treated cells (set at 100%). The measured amounts of calmodulin indicated that there was approximately 3 pg of calmodulin (~1.8 fmoles) per control cell. All three separate experiments showed a decreased calmodulin protein in antisense ODN-treated cells relative to control ODN-treated cells (with a range of 18–43%). Qualitatively similar results were obtained when mRNA levels were examined after antisense and control ODN treatments (Yano, Bell & Van Houten, pers. observ.). A previous experiment has already shown that reduced calmodulin levels in *Paramecium* correlate with reduced chemoresponse [19]. It may be possible to reduce calmodulin levels further using higher doses of ODN or a mixture of ODNs targetted to the calmodulin mRNA. This was not tested but it is already known that increasing by 10-fold (16–32 μM) the amount of ODN microinjected into a cell results in cell death not seen in control cells injected with similar amounts of a control ODN (Hinrichsen,

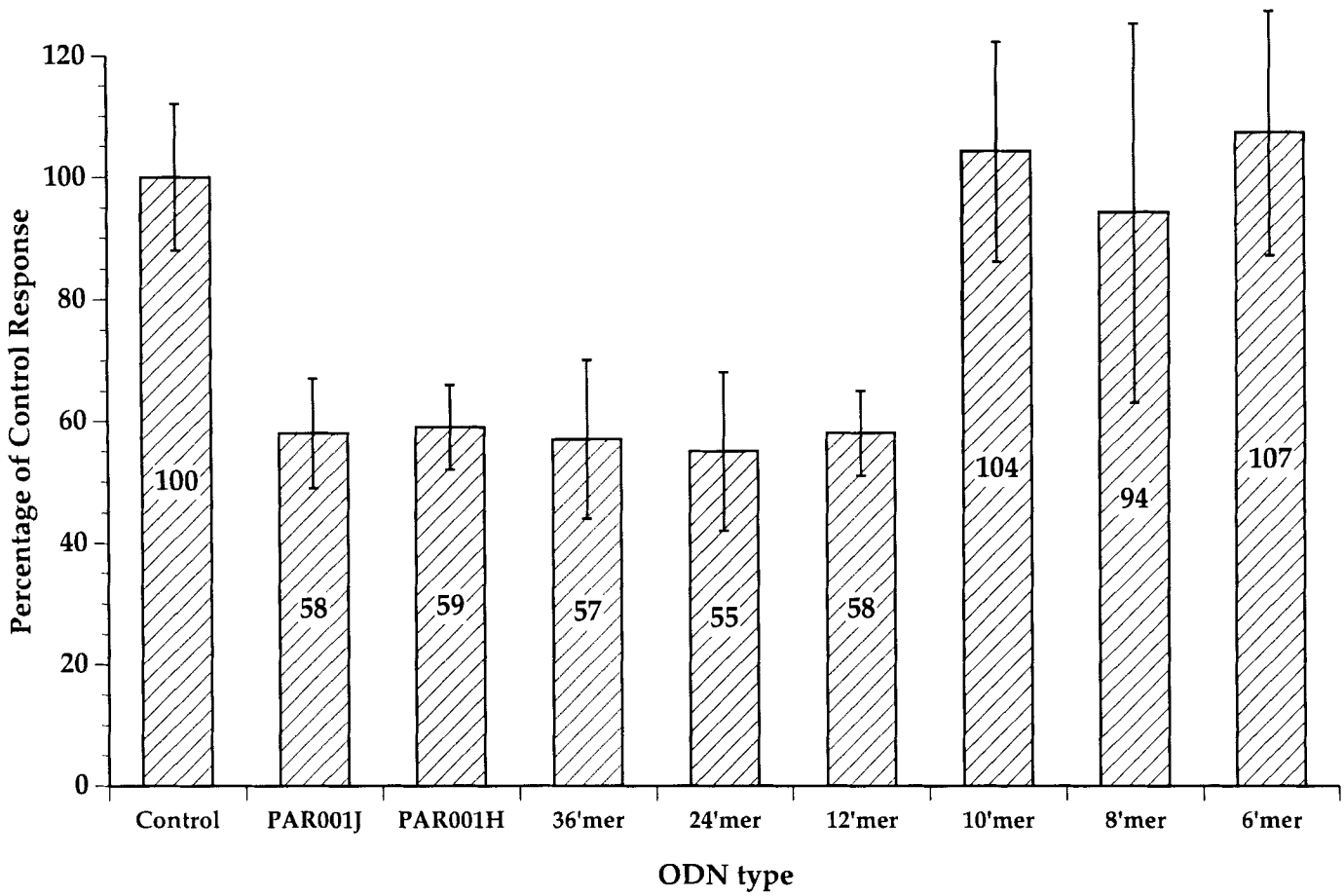

Fig. 4. Effect of oligodeoxynucleotide (ODN) size and 3′ modification upon antisense efficiency in mutant *Cam 1* of *Paramecium*. Cells were electroporated in the presence of 10 μM of different ODNs with the following modifications: PAR001J—3′ hexanol modified, PAR001H—3′ cholesterol modified, or various sized ODNs covalently linked at their 3′ end to hexanol (36′mer through 6′mer). Control ODNs were either PAR501H (for PAR001H) or PAR501J (for all other ODNs). Each experiment was performed three times and the values presented are from a typical experiment. Control cells swam backwards on average between 80–120 s under the conditions used. All values were normalized to the backward-swimming response of the appropriate control-treated cells tested at the same time, reflecting the average percentage-of-control response seen for each treatment expressed as a mean. A normalized value of 100% was used for the control cells with a standard deviation given for all treatments to reflect the amount of variability seen in a typical experiment. After electroporation, the cells were allowed to incubate in 10% exhausted wheatgrass in resting solution at 28° C for 10–12 h and tested in Na/TEA testing solution. The effects seen with PAR001J, PAR001H, ODN 12 bases (12′mer) or larger were significantly different than for the shorter ODN ($p < 0.001$) as judged by the Mann-Whitney *U* Test for significance.

Fraga, & Reed, unpubl. data). This observation is consistent with the hypothesis that reducing calmodulin levels below a certain level is lethal.

DISCUSSION

The major conclusion from the work presented here is that electroporation is an easy and effective method of introducing AS ODN into *Paramecium*. The use of electroporation as a method for introducing AS ODN has been reported before in a study in which the expression levels of the *myc* oncogene in tissue culture cells were down-regulated using electroporated AS ODN [1]. Its use in *Paramecium* can be to replace the technically more challenging method of microinjection. We have examined a number of parameters that are important for effective antisense treatment of *Paramecium* through electroporation. In addition, we have provided some evidence that the antisense ODN treatment described here results in lower levels of the target protein in the cell. Similar reductions in calmodulin levels in *Paramecium* have been previously reported to effect the cell's chemoresponse ability [19].

Key points concerning use of this technique are as follows: a) cells should be in a late-log to early-stationary phase of growth; b) the ODN should be at least 12 bases in size; c) cells began showing significant changes in their behavioral response after 10–12 h at 28° C and continued until approximately 14–20 h later, at which point all cells responded normally to test solutions; and d) cholesterol and hexanol can be used as 3′ blocking groups. We suspect that the exact parameters will vary between different message targets, but this does serve as a useful starting and comparison point.

The experiments to determine minimal ODN length (at 10 μM) and minimum concentration for anti-calmodulin effects used a 3′-hexanol blocking group. This modification was preferred since it slows nuclease degradation in cells but it has little effect on hybridization properties and is not lipophilic. The lipophilic 3′-cholesterol group has an affinity for cellular mem-

branes and proteins and has been shown to have enhanced antisense efficacy when added directly to the medium of cultured mammalian cells [11]. There was no apparent difference between the anti-calmodulin effects of electroporated cholesterol- and hexanol-modified ODN at 10 μM. This implies that both ODNs have equal access to calmodulin mRNA targets.

We also examined the effect of using multiple ODNs directed to the same gene as a means to amplify the effects of the ODNs. We were able to reproduce what previous authors had reported as a synergistic effect [12]. This method resulted in a 1 μM combined dose (when using 2 different ODNs at 0.5 μM each) being effective, a dose that was ineffective when using just one ODN (at 1 μM) targeted to the mRNA of interest.

The use of synergistically-acting AS ODN and different 3′ blocking groups can allow one to address the persistent problem of nonspecific effects when using antisense ODN. For example, by utilizing a combination of synergistically acting ODN and a variety of 3′ blocking groups one can reduce the chance of being misled by nonspecific effects. Since the use of synergistic ODNs will only be effective at the reduced dose, if both ODNs are acting on the same target, the potential for nonspecific effects (which are dose-dependent) is greatly reduced. Concerns about nonspecific effects caused by the use of a particular 3′-blocking group can be addressed by employing different 3′-blocking groups. By testing different ODNs that bind to the same target, one can be more confident of measuring specific and meaningful effects.

In summary, the use of electroporated AS ODN can be relied upon to quickly identify potential genes involved in a cellular process of interest, which can then be further analyzed by other means. It is important to emphasize that this approach is an effective qualitative tool but it should not be considered a precise quantitative tool due to a nonuniform dosing of the treated cells, which was directly measured here by the use of a fluorescently-tagged ODN. Nonuniform dosing was also suggested by 8 to 29% of the cells being unaffected by the AS treatment over the time-course of the experiment. However, we feel this tool can be used to identify genes that are involved in the regulation of many cellular activities in *Paramecium*. Preliminary experiments that look at the role of protein phosphatases in the regulation of cell motility support this approach (Fraga, Russell, & Hinrichsen, unpubl.).

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