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Efficient transformation of *cam*^{2}, a behavioral mutant of *Paramecium tetraurelia*, with the calmodulin gene

(telomere/macronucleus)

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ABSTRACT **An Ile-136 → Thr substitution in calmodulin reduces the Ca^{2+}-dependent K^+ currents of *cam*^{2}, a behavioral mutant of *Paramecium tetraurelia*, and renders it overly susceptible to $BaCl_2$. DNA fragments carrying the wild-type *CAM* gene injected into *cam*^{2} macronuclei reverted these phenotypes in the clonal descendants of the recipients. *Tetrahymena* telomeric sequences, added *in vitro* to the fragment termini before injection, enhanced the efficiency and quality of transformation. Five times 10^4 copies of such fragments consistently restored the phenotypes to near normal; even 10^3 or fewer copies could still effect weak transformation. The restored phenotypes were stable for >20 fissions in many clones and were lost after autogamy. We examined the fate of the injected fragments in the transformed clones and discuss the possible application of this efficient transformation in the cloning of other genes of *P. tetraurelia*.**

The development of *Paramecium* molecular biology is dependent upon the ability to efficiently transform mutants and thereby to identify and clone genes that are altered in those cells. Tondravi and Yao (1) succeeded in ciliate transformation by injecting mutant rRNA-encoding DNA into the macronuclei of *Tetrahymena*. Godiska *et al.* (2) transformed a mutant of *Paramecium tetraurelia* that is incapable of expressing the immobilization antigen A by injecting 10^6 copies of the cloned wild-type immobilization antigen A gene. Clonal descendants of recipient paramecia appear to linearize the circular plasmids in a sequence-nonspecific manner and replicate them in a telomere-bracketed form (2, 3). Telomeres are known to protect eukaryotic chromosome ends (4, 5). The telomeric sequence required for this protection is not strictly species specific: artificial chromosomes with *Tetrahymena* telomeres are stable in yeast (6); linear plasmids with yeast telomeres are stable in *Stylonychia* (7).

Paramecia respond behaviorally to various stimuli (8). Among behavioral mutants of *P. tetraurelia* are mutants of the calmodulin structural gene (*CAM*) (9). These mutants exhibit either prolonged (pantophobiac subtype) or abbreviated backward swimming (fast 2 subtype) to certain stimuli. One such allele of the pantophobiac subtype, *cam*^{2}, has an isoleucine → threonine substitution at position 136 in the fourth Ca^{2+}-binding pocket of calmodulin (9, 10) (Fig. 1*A*). This substitution causes a loss of the Ca^{2+}-dependent K^+ currents (11). Pleiotropic effects of this mutation include the pantophobiac behavior, mating incompetence, and a higher sensitivity to $BaCl_2$ than wild type (9, 12). We have chosen the *cam*^{2} mutant as a test case for the development of efficient transformation of *Paramecium* behavioral mutants. Furthermore, we have investigated the importance of telomeric sequences added *in vitro* to plasmids carrying *CAM* and the possible role of these telomeric sequences in protecting injected DNA. We discuss application of this strategy to identify and clone other genes that can phenotypically complement other *Paramecium* behavioral mutants.

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MATERIALS AND METHODS

Stocks and Culture. All *P. tetraurelia* stocks used had the *nd6/nd6* background mutation (13). Cells used for injection (*cam*^{2}) also had the *cam*^{2}/*cam*^{2} mutation (9). Paramecia were cultured in medium M supplemented with *Enterobacter aerogenes*. This medium contains 100 mg of glucose per liter, 100 mg of Casamino acids per liter, 10 mg of stigmasterol per liter, 7.5 mg of phenol red per liter, 0.5 mM K_2HPO_4, 0.2 mM $MgSO_4$, 0.2 mM $CaCl_2$, and 5 mM Hepes/NaOH (pH 7.0–7.5).

Microinjection. DNA suspended in TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) was injected using a Narishige microinjection device (IM-200) in a manner similar to that of Godiska *et al.* (2). Except for one set of control cytoplasmic injections, we injected the DNA into macronuclei. The number of plasmids injected into the macronucleus was calculated on the basis of a maximal 20-pl delivery (14, 15).

Test of Survival in $BaCl_2$. Injected cells were allowed to multiply in supplemented medium M with no selective pressure. As diagramed in Fig. 2*A*, 20 cells from each clone were transferred into medium M laced with 0.5 mM $BaCl_2$ and 10 mM NaCl (Ba^{2+} survival test solution). Uninjected *cam*^{2} cells die in $BaCl_2$ solutions (16) and in this solution usually died within 24 hr, whereas wild-type cells survived and eventually multiplied to >100 cells in 3 days. The survivability in $BaCl_2$ for 20–30 hr was used to grade the quality of the transformation as follows: grade 0, <6 cells survived (usually none); grade 1, 6–20 cells survived; grade 2, >20 cells survived but multiplied poorer than wild type; grade 3, >40 cells survived (similar to wild type).

Behavioral Test. Clonal descendants of injected cells (Fig. 2*A*) were transferred into a K^+/Ca^{2+} solution (4 mM KCl/1 mM $CaCl_2$/1 mM Hepes/0.01 mM EDTA, pH 7.2). After 5 min, four or more cells per clone were individually transferred under a dissecting microscope into 5 mM tetraethylammonium chloride/5 mM NaCl added to the K^+/Ca^{2+} solution without its KCl. The duration of backward swimming immediately upon transfer was timed using a stopwatch.

Voltage Clamp. The techniques used to elicit and analyze membrane currents of *Paramecium* under voltage clamp have been described (17).

Recombinant DNA Methods. Standard molecular biological techniques were used (18) except those below. Total *Paramecium* DNA was extracted as described (19) with slight modifications. The plasmid pNTTEL was constructed by ligating a 3.2-kb *Xho* I fragment (containing the *Tetrahymena* rRNA-encoding DNA telomere sequences) from pYAC3 (American Type Culture Collection, no. 37502) at the *Xho* I site of pNT1 (gift of K. McGrath and M. Gorovsky). The *CAM* sequence inserted in pNTTEL to create pCAMTEL (Fig. 1*B*) was generated by a PCR. The template of this PCR was a plasmid carrying the *CAM* open reading frame with 5′ and 3′ flanks (9), and the primers were oligonucleotides

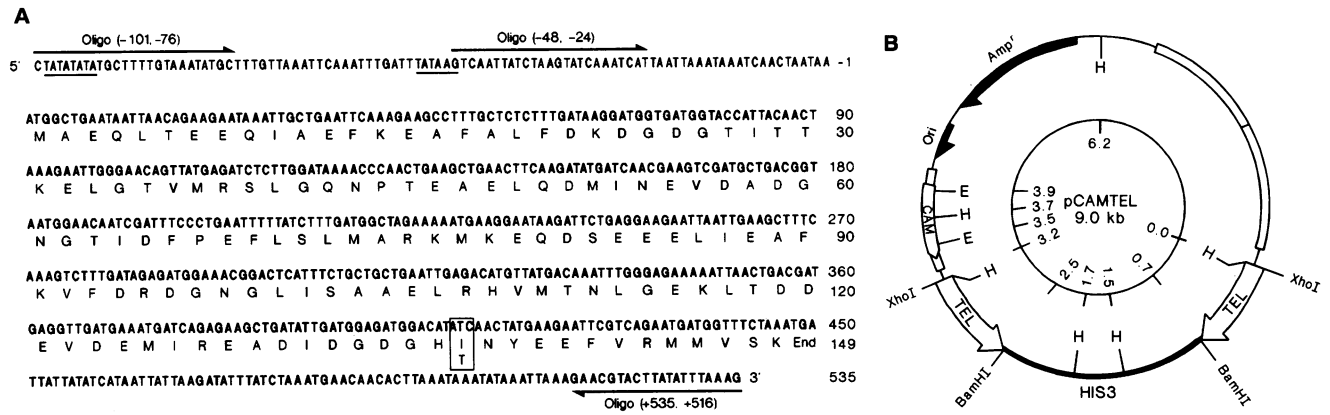

FIG. 1. (*A*) Nucleotide sequence of wild-type *P. tetraurelia* calmodulin gene. Arrows mark oligonucleotides that primed PCRs used in plasmid constructions. Putative TATA sequences are underlined. The Ile → Thr substitution in *cam*^{2} is boxed. (*B*) Map of pCAMTEL. CAM, the *CAM* gene, showing the coding region and flanks; TEL, *Tetrahymena* telomeres, oriented as arrows; Ori, bacterial origin of replication; Amp^r, β-lactamase gene; thin line, pUC-derived sequence; HIS3, yeast *HIS3* gene. *Bam*HI, *Xho* I, *Hin*dIII (H), and *Eco*RI (E) restriction sites are shown. kb, Kilobases.

(University of Wisconsin Biotechnology Center) designated Oligo (−101, −76) and Oligo (+535, +516) (Fig. 1*A*). The PCR product, including putative TATA boxes and the putative poly(A) signal, was ligated to a *Pvu* II site of pNTTEL. The *cam*^{2} gene sequence in pcam^{2}TEL was also generated by a PCR using *cam*^{2} total DNA as template, primed as above, and the PCR product ligated as above. The plasmid pCAMΔ5′TEL was constructed by ligating the product generated by a PCR primed with Oligo (−48, −24) and Oligo (+535, +516) (Fig. 1*A*) on the *CAM* template described above. This product was inserted into pNTTEL as above. PCR amplifications of *CAM* or *cam*^{2} genes were as described (9). Probes in blot hybridizations were pUC19 (BRL) (pUC probe), a pUC18-derived plasmid carrying the yeast *HIS3* gene (*HIS*3 probe, gift of J. Hill), and the *CAM* PCR product (*CAM* probe). These probes were labeled (specific activity ≈ 5×10^8 cpm/μg) by primer extension using an oligonucleotide labeling kit (Pharmacia). Hybridizations were performed in 0.4 M Na_3PO_4, pH 7.2/10 mM EDTA/7% SDS/13% dextran sulfate at 65°C. Washes were performed at 65°C in 0.2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate).

RESULTS

Injection of *CAM*-Bearing Fragments Restores Wild-Type Phenotypes in *cam*^{2}. This report includes results from >40 batches of injections comprising 5–60 cells each. Twenty picoliters of pCAMTEL at 20 μg/ml was the maximal delivery into each macronucleus and was equivalent to about 50,000 molecules. Injection of *Bam*HI-digested pCAMTEL (Fig. 1*B*) transformed 65% of *cam*^{2} recipients that survived the injection (n = 266). As injection techniques improved, we approached 100% transformation with a comparable amount of DNA (Fig. 2*B*). Transformed clones varied only slightly in their $BaCl_2$ survivability: among the early batches of transformants, >80% were grade 3 (n = 172) and >90% were grade 3 in the later batches (n = 25). To test whether *cam*^{2} transformation was indeed due to the *CAM* gene, we injected a *Bam*HI digest of pNTTEL, a plasmid lacking this gene (100 μg/ml, about 250,000 copies) and found no transformants (n = 30). Injection of a *Bam*HI digest of pcam^{2}TEL, a plasmid identical to pCAMTEL except for the Ile-136 → Thr substitution of *CAM*, at 150,000 copies per nucleus, also failed to transform *cam*^{2} (n = 28). Injection of 60,000 copies of *Bam*HI-digested pCAMΔ5′TEL, lacking the *CAM* 5′ flank containing a putative TATA box, again resulted in no transformation (n = 31). Injection of 50,000–800,000 copies per cell of *Bam*HI-digested pCAMTEL into the cytoplasm also failed to transform *cam*^{2} cells (n = 17). Cells from clones that had been transformed, as judged by $BaCl_2$ survival, were tested for their response to tetraethylammonium chloride/NaCl solution (Fig. 2*A*). Wild-type cells showed a continuous backward swimming for 5.8 ± 8.5 s (mean ± SD, n = 15), whereas untransformed *cam*^{2} swam backward for 112.0 ± 29.2 s (n = 12). Grade 3 *cam*^{2} transformants swam backward

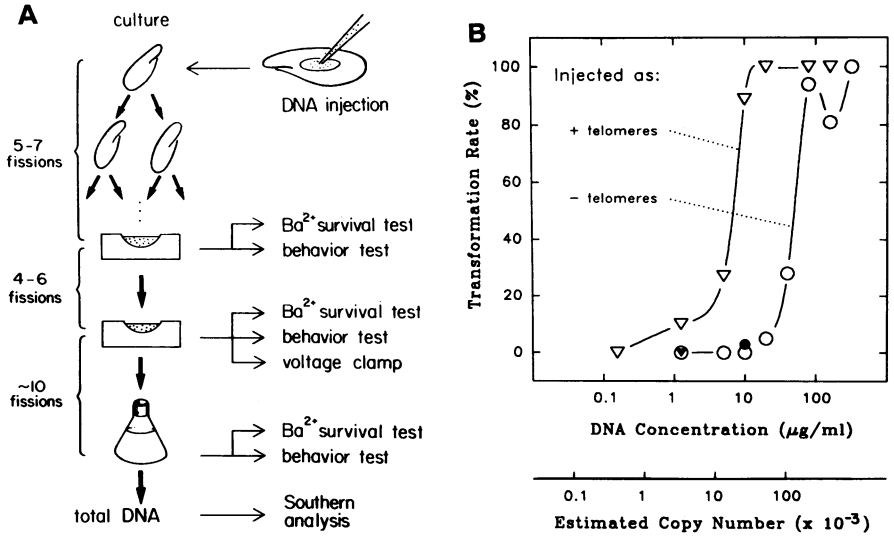

FIG. 2. (*A*) Diagram of transformation and testing protocol. (*B*) Transformation rate (based on $BaCl_2$ survivability) in relation to the amount of DNA injected. Different DNA concentrations were generated by sequential 2-fold dilutions of *Bam*HI-digested (△, ▲) or *Xho* I-digested (○, ●) DNA stocks of >600 μg/ml with TE buffer (△, ○) or with TE buffer containing pNTTEL (100 μg/ml) cut with *Bam*HI (▲) or *Xho* I (●). Each point denotes a transformation rate based on 15–33 injections, except for the *Bam*HI-digested sample (n = 4) and *Xho* I-digested sample (n = 10) of the highest concentrations.

for 17.4 ± 8.6 s (n = 27), like the wild type. Grade 2 and grade 1 transformants exhibited intermediate responses (42.9 ± 21.9 s, n = 11). Descendants of injected cells that were not transformed (grade 0) behaved like the uninjected *cam*^{2} mutant (105.6 ± 29.0 s, n = 25). Thus, the tetraethylammonium chloride/NaCl solution-induced behavior was strongly correlated with the $BaCl_2$ survivability grades. These two phenotypes presumably reflected various transmembrane ion currents. Two-electrode voltage clamp records showed that the Ca^{2+}-dependent K^+ current activated during depolarization (Fig. 3 *A* and *C*) and the one activated upon hyperpolarization (Fig. 3 *B* and *D*), both greatly reduced in the *cam*^{2} mutant, were restored fully in grade 3 transformants (n = 29).

Less than 10^4 Copies of pCAMTEL Are Needed to Transform *cam*^{2}. Dilution experiments were performed to determine how many copies of *Bam*HI-digested pCAMTEL were needed to achieve transformation. Compared to the grade 3 $BaCl_2$ survival score obtained in 80% or more of transformants upon injection of 50,000 copies (20 pl of 20 μg/ml DNA), 5000 copies (2 μg/ml) effected only 9% transformation (n = 69) of quality lower than grade 2 on average. With 500 copies (0.2 μg/ml), only 5%, grade 1, transformation was observed (n = 39). No transformation was observed when 50 copies (0.02 μg/ml) were injected (n = 46). There was a clear correlation between the number of *CAM*s injected per nucleus and the rate and quality of transformation. We conclude that $<10^3$ copies of *Bam*HI-digested pCAMTEL is required to transform *cam*^{2}.

Telomeres Enhance Transformation. Unlike *Bam*HI, *Xho* I digestion of pCAMTEL releases a *CAM*-bearing fragment, which is free of telomeres (Fig. 1*B*). Injection of *Xho* I digests of pCAMTEL (50,000 copies) gave only 13% transformation (grade highly variable). This approximated the quantity and quality of transformation achieved using 5000 copies of the *Bam*HI digest of the same plasmid, where *CAM* was bracketed by telomeres (Fig. 1*B*). A comparison of transformation potency of the telomere-bracketed vs. telomere-free *CAM* fragments over a large range of DNA concentrations showed that 10 times more of the latter was needed to effect the same transformation as the former (Fig. 2*B*).

Injected CAM Fragment Replicates Autonomously. Total DNA was isolated from *cam*^{2} cells transformed with *Bam*HI digests of pCAMTEL and was examined by DNA blot hybridization without further digestion. A discrete species of DNA at 7.2 kb was found to hybridize to the *CAM* probe (Fig. 4*A*, lanes 2–6) and to the pUC probe (Fig. 4*A*, lanes 9–13). This DNA comigrated with the 7.2-kb *CAM*-carrying *Bam*HI fragment (Fig. 4*A*, lane 7) contained in the injected *Bam*HI digest (Fig. 1*B*, inner circle, from the 2.5-kb mark clockwise to the 0.7-kb mark). These blots therefore show the presence of *CAM*-carrying fragments of the size of the input DNA in the transformed clonal descendants of the injected cells. The intensity of the hybridizing DNA, reflecting the plasmid copy number, corresponded roughly to the quality of the transformed clones, as graded by $BaCl_2$ survivability (Fig. 4*A*). The *CAM* probe also recognized sequences in a higher molecular weight region of these undigested total DNAs (Fig. 4*A*, lanes 1–6, asterisk), presumably the native *cam*^{2}-bearing chromosomes. The absence of any hybridization with the pUC probe to the higher molecular weight region (Fig. 4*A*, lanes 9–13) suggests that no detectable recombination had occurred between the foreign fragments and the genome. It therefore appears that this 7.2-kb *CAM*-carrying *Bam*HI fragments replicated, with no detectable variance in size, autonomously in the transformed *cam*^{2} nuclei. As expected, pCAMTEL and transformed phenotypes were lost following autogamy after >20 fissions (ref. 20; unpublished results).

Injected Telomere-Free CAM Fragments Are Modified in Transformants. In contrast to the discrete 7.2-kb *CAM*-bearing fragment detected in cells transformed with *Bam*HI-digested pCAMTEL (Fig. 4*B*, lanes 1 and 2), *CAM* probe hybridized with DNA more heterogeneous in size from cells transformed with *Xho* I-digested pCAMTEL (Fig. 4*B*, lanes 3 and 4). Although the major hybridizing DNA species in these cases comigrated with a 5.8-kb fragment (Fig. 4*B*, lane 9), expected of the *Xho* I-digested pCAMTEL (Fig. 1*B*, 3.2-kb clockwise to 0.0-kb mark), hybridizing DNA also smeared upward and downward from the 5.8-kb region. Furthermore, in every clone examined, at least two discrete species of hybridizing DNA migrating slower than the 5.8-kb band could be discerned against the background. Such hybridizing DNA was never seen in blots from clones transformed with the *Bam*HI digest of pCAMTEL.

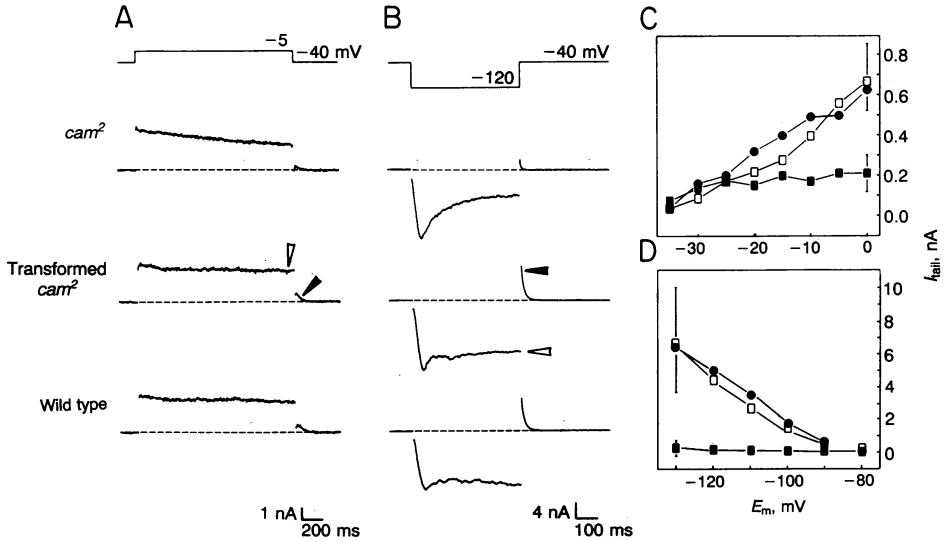

FIG. 3. Restoration of two distinct Ca^{2+}-dependent K^+ currents in transformed *cam*^{2} cells. (*A*) *cam*^{2} trace, depolarization of *cam*^{2} (from −40 mV to −5 mV) elicits an outward K^+ current. Transformed *cam*^{2} trace, depolarization of a grade 3 *cam*^{2} transformant additionally evokes a slower, outward, Ca^{2+}-dependent K^+ current (open arrowhead). This current is also manifested as a slow outward tail current (filled arrowhead) upon returning to the holding potential, the magnitude of which is comparable to that observed in the wild-type trace. (*B*) *cam*^{2} trace, hyperpolarization (from −40 mV to −120 mV) elicits K^+ currents without the Ca^{2+}-dependent component. Transformed *cam*^{2} trace, the Ca^{2+}-dependent K^+ current (arrow heads) is restored in a grade 3 transformant and is indistinguishable from that of the wild type (wild-type trace). (*C*) Amplitude of Ca^{2+}-dependent K^+ tail currents (I_{tail}), plotted as a function of step membrane potential (E_m), as determined 20 ms following a return from depolarization to the holding potential (*cam*^{2}, filled squares, n = 8; transformed *cam*^{2}, grade 3, open squares, n = 11; wild type, filled circles, n = 6). Standard deviations are only given for one point each; other values are similarly variable. (*D*) Amplitude of Ca^{2+}-dependent K^+ tail currents (I_{tail}), plotted as a function of membrane potential (E_m), immediately following a return from hyperpolarization to the holding potential by back-extrapolation of fitted exponentials, as described (17). (*cam*^{2}, filled squares, n = 15; transformed *cam*^{2}, grade 3, open squares, n = 18; wild type, filled circles, n = 10).

As described, pCAMTEL was digested with either *Bam*HI or *Xho* I prior to microinjection. To examine the fate of the termini generated by these digestions, we isolated total DNA from the transformants about 20 fissions after injection and digested the DNA with either *Hin*dIII or *Eco*RI (Fig. 4*C*). All of the predicted pCAMTEL restriction fragments were detected by hybridization to the pUC and *CAM* probes, except for those with little or no sequence homology to the probes (e.g., *HIS3* and TEL sequences). Of particular interest were those fragments representing termini of the injected DNA. The *Eco*RI digest revealed a discrete 5.7-kb DNA species (Figs. 4*C*, lanes 1 and 2 and 1*B*, 3.9-kb to 0.7-kb mark) from cells transformed with *Bam*HI-digested pCAMTEL. By contrast, *Eco*RI digestion of DNA from cells transformed with *Xho* I-digested pCAMTEL revealed that the corresponding terminus of the injected DNA was heterogeneous in size, centered around the expected 5.0-kb size (Figs. 4*C*, lanes 3 and 4 and 1*B*, 3.9-kb to 0.0-kb mark).

Analysis of *Hin*dIII digests of transformant total DNA was consistent with this finding. Two sets of immediately adjacent *Xho* I and *Hin*dIII sites are found on pCAMTEL (Fig. 1*B*, 0.0-kb and 3.2-kb marks). Fragments released by *Hin*dIII digestion of total DNA from cells transformed with *Bam*HI-digested pCAMTEL included two restriction fragments, one of ≈0.4 kb and one of 2.8 kb (Fig. 4*C*, lanes 5 and 6), which can serve as size standards for two of the *Hin*dIII–*Xho* I fragments (Fig. 1*B*, 3.2-kb to 3.7-kb mark and 6.2-kb to 0.0-kb mark), both of which were terminal fragments at the time of injection of *Xho* I-digested pCAMTEL. Most striking was the heterogeneity of the smaller of these two terminal fragments (Fig. 4*C*, bracket, lanes 7 and 8), detected in cells transformed with *Xho* I-digested pCAMTEL. These fragments were up to 400 bp larger than the 442-bp fragment (Fig. 4*C*, arrow), which would have been the terminus if unmodified. Examination of the larger 2.8-kb terminal fragment (Fig. 1*B*, 6.2-kb to 0.0-kb mark) suggested similar modifications. It should be noted that fragments from internal sequences (not termini at the time of injection) appeared homogeneous, whether from transformants injected with *Bam*HI-digested or *Xho* I-digested pCAMTEL.

Small Fragments Can Be Lost Completely. In addition to the *CAM*-bearing fragment, the *Bam*HI digest of pCAMTEL included the 1.8-kb *HIS3* fragment without telomeres (Fig. 1*B*, 0.7-kb to 2.5-kb mark). Similarly, the *Xho* I digest of pCAMTEL contained the 3.2-kb *HIS3* fragment bracketed by telomeres in the reverse orientation (Fig. 1*B*, 0.0-kb to 3.2-kb mark). When probed with *HIS3*, the 1.8-kb fragment was never detected (Fig. 4*B*, lanes 5 and 6, open triangle), whereas the 3.2-kb fragment was present in every transformed clone examined and also showed size heterogeneity (Fig. 4*B*, lanes 7 and 8, filled triangles).

DISCUSSION

We report here that a cluster of electrophysiological, behavioral, and survival defects associated with the Ile-136 → Thr

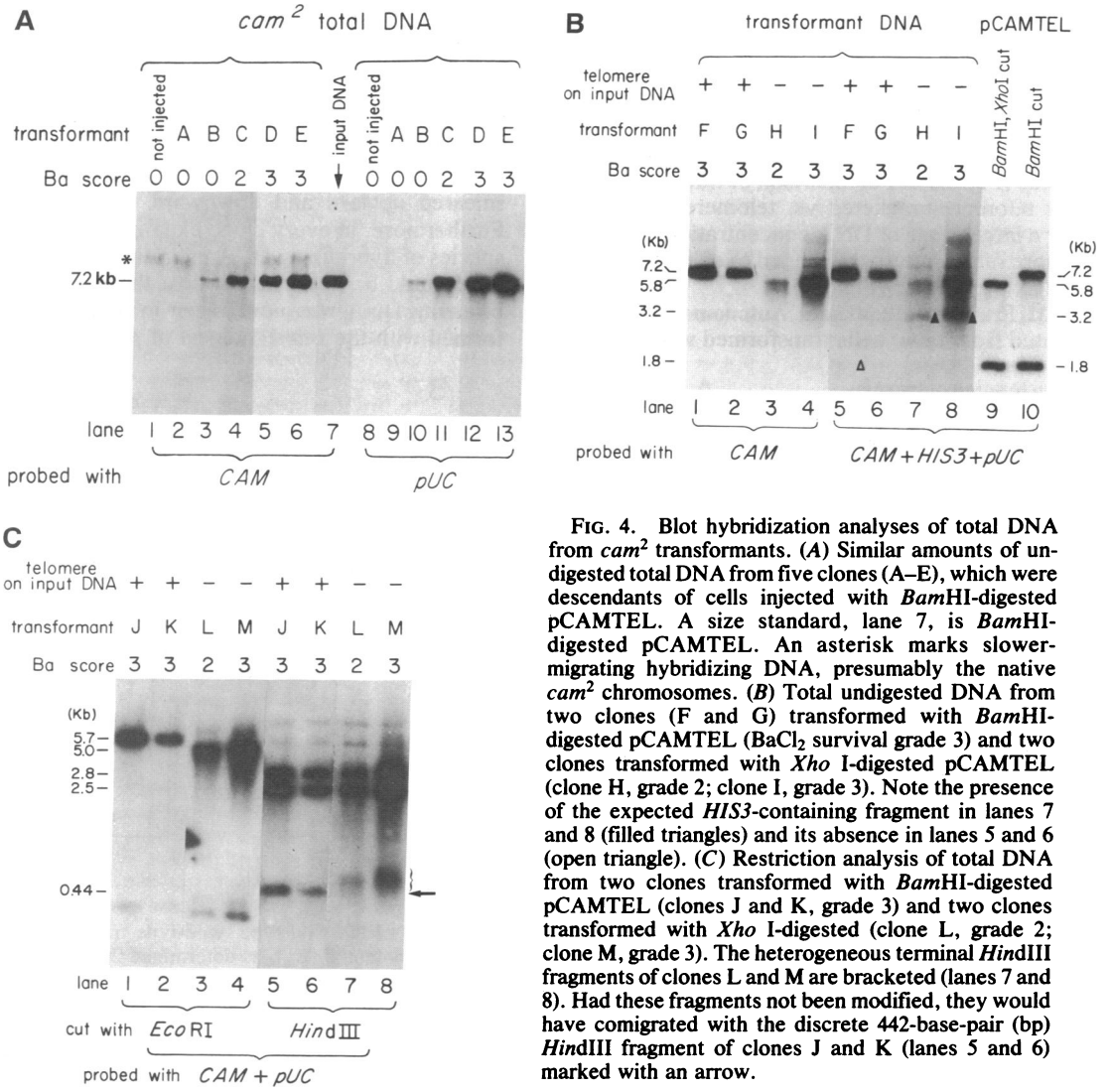

FIG. 4. Blot hybridization analyses of total DNA from *cam*^{2} transformants. (*A*) Similar amounts of undigested total DNA from five clones (A–E), which were descendants of cells injected with *Bam*HI-digested pCAMTEL. A size standard, lane 7, is *Bam*HI-digested pCAMTEL. An asterisk marks slower-migrating hybridizing DNA, presumably the native *cam*^{2} chromosomes. (*B*) Total undigested DNA from two clones (F and G) transformed with *Bam*HI-digested pCAMTEL ($BaCl_2$ survival grade 3) and two clones transformed with *Xho* I-digested pCAMTEL (clone H, grade 2; clone I, grade 3). Note the presence of the expected *HIS3*-containing fragment in lanes 7 and 8 (filled triangles) and its absence in lanes 5 and 6 (open triangle). (*C*) Restriction analysis of total DNA from two clones transformed with *Bam*HI-digested pCAMTEL (clones J and K, grade 3) and two clones transformed with *Xho* I-digested (clone L, grade 2; clone M, grade 3). The heterogeneous terminal *Hin*dIII fragments of clones L and M are bracketed (lanes 7 and 8). Had these fragments not been modified, they would have comigrated with the discrete 442-base-pair (bp) *Hin*dIII fragment of clones J and K (lanes 5 and 6) marked with an arrow.

calmodulin mutation (cam^2) can be corrected in clonal descendants of cells injected with *CAM*-bearing DNA fragments. cam^2 cells also mate poorly; even this defect was complemented in the transformants (unpublished results). By the same means, we were also able to transform cam^{13}, an allelic variant with a different cluster of defects (ref. 9; unpublished results). Also, we report here that the addition of telomeric sequences to *CAM*-bearing fragment *in vitro* prior to injection stabilizes the injected DNA and enhances efficiency of cam^2 transformation.

Godiska *et al.* (2) and Gilley *et al.* (3) showed that circular DNA injected into the ciliate macronucleus is linearized and elongated by the addition of telomere-like sequences in the transformants. We also found that injection of circular pCAMTEL could effect transformation (unpublished results). Furthermore, this report shows that injected linear *CAM*-bearing fragments not bracketed by telomeric sequences (*Xho* I digest of pCAMTEL) propagated in the transformed clones as molecules heterogeneous in size (Fig. 4*B*). Since these descendant fragments can be shorter or longer than the injected fragments, they might have been subjected to two competing effects: exonuclease attack and telomerase-mediated elongation. The mechanisms for elongation and fixation are unknown but presumably include the addition of telomeric sequences (2, 3, 21). Some fragments appeared larger than 5.8 kb by several kilobases, though variable from clone to clone, and were replicated at fixed lengths (Fig. 4*B*). Concatenation of injected DNA fragments unprotected by telomeric sequences in the macronucleus of *Paramecium primaurelia* has recently been reported (21). Although not investigated, it is possible that the discrete higher molecular weight DNA species detected in cells transformed with *Xho* I-digested pCAMTEL represent concatemers. Interestingly, such concatenation of fragments bracketed by telomeric sequences was not detected in our study (Fig. 4*A*, lanes 4–6 and 11–13) or in *P. primaurelia* (21).

Ten times more of the telomere-free *CAM* fragment is needed to effect the same degree of transformation as the telomere-bracketed fragment (Fig. 2*B*). This result may mean that 90% of the *CAM* genes in the telomere-free fragments have been destroyed or damaged, presumably by exonuclease attack, before they become protected, possibly by telomere capping. This is consistent with the observed low percentage and low grades of transformation with these telomere-free fragments, since low-grade transformations appear to correspond to small numbers of intact *CAM* genes remaining (Fig. 4*A*). In addition to protection, telomeres may promote replication (21) and the unreplicated or underreplicated fragments deprived of telomeres may be lost simply by dilution during clonal expansion. In any event, by performing tasks *in vitro* (linearization and telomere addition) otherwise performed less effectively by the macronuclear machinery, transformation efficiency is enhanced; 10^3 to 10^5 *CAM* copies per cell appear to be maintained in weak to strong transformants, as estimated by densitometry of autoradiograms (unpublished results). The numbers of the maintained copies appear to parallel those of the injected copies, indicating replication and no significant amplification. Though not likely, it is possible that the *CAM*-bearing fragment released by *Bam*HI is more transformation potent than that released by *Xho* I, not because of the nature of the telomeric sequence but simply because of the greater distance (>700 bp) to the terminus occupied by the telomeric sequences (Fig. 1*B*). A unique *Eco*RV site, which is >2 kb from *CAM*, exists in pCAMTEL. Injection of an *Eco*RV digest of pCAMTEL (20 pl at 20 μg/ml) did not transform cam^2 better than the *Xho* I digest did (unpublished results). Thus, it appears that telomeres on the input DNA increase its transformation potency not simply as "stuffer-sequences" that separate the gene from the free ends.

Unlike the study of the immobilization antigen A expression mutant, where injections of 10^6 copies of the wild-type gene effect the transformation (2, 3, 21), we found that only 5×10^4 telomere-carrying *CAM* fragments gave frequent and strong transformation of cam^2. Even 10^3 or fewer copies yielded detectable transformants. Furthermore, these numbers are likely overestimates (see *Materials and Methods*). Injection of 10^3 copies of *CAM* can apparently complement successfully the 10^3 cam^2 genes native to the cam^2 macronucleus. This result agrees with the genetic observation that the cam^2 allele is recessive to *CAM* in the heterozygote (16). We have successfully transformed cam^2 cells by injecting a sample of total wild-type DNA containing as few as 10^2 *CAM* genes in their natural context (unpublished results). This context may include enhancer sequences not contained in pCAMTEL, and they may enhance the expression of *CAM* in cam^2 cells. A study of the upstream 5′ flank of the *CAM* gene is necessary. That $<10^3$ copies of a telomere-bracketed gene can transform a mutant makes hopeful the isolation of other *Paramecium* genes through sublibrary sorting and expression cloning. In addition, *Paramecium* artificial chromosomes, perhaps of design similar to pCAMTEL, can be developed and employed, especially when transformation en masse becomes practical.

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