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K^+-Channel Transgenes Reduce K^+ Currents in Paramecium, Probably by a Post-translational Mechanism

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ABSTRACT

PAK11 is 1 of more than 15 members in a gene family that encodes K^+-channel pore-forming subunits in *Paramecium tetraurelia*. Microinjection of *PAK11* DNA into macronuclei of wild-type cells results in clonal transformants that exhibit hyperexcitable swimming behaviors reminiscent of certain loss-of-K^+-current mutants. *PAK2*, a distant homolog of *PAK11*, does not have the same effect. But *PAK1*, a close homolog of *PAK11*, induces the same hyperexcitability. Cutting the *PAK11* open reading frame (ORF) with restriction enzymes before injection removes this effect entirely. Microinjection of *PAK11* ORF flanked by the calmodulin 5′ and 3′ UTRs also induces the same hyperexcitable phenotype. Direct examination of transformed cells under voltage clamp reveals that two different Ca^{2+}-activated K^+-specific currents are reduced in amplitude. This reduction does not correlate with a deficit of *PAK11* message, since RNA is clearly produced from the injected transgenes. Insertion of a single nucleotide at the start of the *PAK11* ORF does not affect the RNA level but completely abolishes the phenotypic transformation. Thus, the reduction of K^+ currents by the expression of the K^+-channel transgenes reported here is likely to be the consequence of a post-translational event. The complexity of behavioral changes, possible mechanisms, and implications in Paramecium biology are discussed.

ALL known K^+ channels have similar features: each consists of four pore-forming subunits that form the K^+ filter and aqueous pathway across the membrane (Doyle *et al.* 1998). Some channels are homotetramers, especially those overexpressed heterologously from a single subunit gene, whereas others appear to be heterotetramers. Jegla and Salkoff (1995) first described two K^+-channel sequences from a *Paramecium tetraurelia* genomic library. We have expanded this K^+-channel subfamily by an additional five members (Y. Saimi, unpublished data). Meanwhile, the Paramecium genome-sequencing project (Dessen *et al.* 2001) has revealed nine more sequences, all unique. As of this writing, there is evidence for at least 16 such K^+-channel subunit genes in *P. tetraurelia*, and this is clearly an underestimate, since only a small fraction (<5%) of the Paramecium genome has been sequenced. In Paramecium, at least seven K^+-specific currents that differ in gating, kinetics, activation, and inactivation have been recognized electrophysiologically (Saimi and Kung 1987; Preston *et al.* 1990a; R. R. Preston, unpublished data). How so many K^+-channel genes are expressed and logistically deployed to different structures and with different functions, all within a single cell, will be a challenge to understand.

In an attempt to identify their role *in vivo*, we have examined the behavioral and electrophysiological effects of expressing individual K^+-channel subunit transgenes in macronuclei of paramecia. In two out of the three transgenes studied, we have encountered a phenomenon resembling silencing. Injection of certain full-length Paramecium K^+-channel genes (*PAK*s; Jegla and Salkoff 1995; Ling *et al.* 1998) elicited hyperexcitable behaviors similar to those of pantophobiac, a K^+-channel loss-of-function mutant (Saimi *et al.* 1983). Surprisingly, our molecular analyses indicate a post-translational instead of a post-transcriptional mechanism.

MATERIALS AND METHODS

Stocks and cultures: *P. tetraurelia* stocks 51s (+/+; Sonneborn 1970) and nd6 (*nd6/nd6*; Lefort-Tran *et al.* 1981) were cultured at 23° or 28° in a growth medium of buffered wheat grass inoculated with *Enterobacter aerogenes* (Sonneborn 1970). nd6 cells are behaviorally wild type, albeit they cannot discharge their trichocysts. Since these mutant cells survive the trauma of macronuclear microinjection better than the wild-type 51s (Kim *et al.* 1992; Haynes *et al.* 2000), only cell lines homozygous for the *nd6* mutation were used as the recipient for microinjection.

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Preparation of total DNA and total RNA from Paramecium: Standard molecular biology techniques were followed (Sambrook *et al.* 1989; Ausubel *et al.* 1999). Total DNAs were extracted from 51s cultures and descendants of microinjected nd6 cells as previously described (Haynes *et al.* 1995). To obtain RNA, cells from 51s, nd6, or descendants of uninjected and microinjected cell lines were harvested and washed once in 10 mm Tris buffer, pH 7.5. Approximately 125-μl aliquots of cells (1×10^5 cells) were lysed in 375 μl of TRI-REAGENT (Molecular Research Center, Cincinnati) in the presence of RNase-free glass beads (Betermier *et al.* 2000). The total RNA of each sample was then purified according to the protocol provided by the manufacturer as described previously (Haynes *et al.* 2000).

Cloning of *PAK1*, *PAK11*, and *PAK2*: A complete *Xba*I digest of total Paramecium DNA was probed with a ^{32}P-labeled 0.85-kb fragment of *PAK1* DNA (Jegla and Salkoff 1995) (5′ ATGCTCTCAATAAAG . . . ACACTCACACTTGGC 3′; see GenBank entry). DNAs corresponding in size to the 4.5-kb band detected in Southern blots were cloned into the *Spe*I site of Bluescript II KS$^+$ (pBS; Stratagene, La Jolla, CA) to form a partial library. The pBS-PAK11 used in the present study was among the positive clones identified on colony lifts by the *PAK1* probe. It was consequently purified and sequenced. When a ^{32}P-labeled 0.8-kb fragment of *PAK2* (Jegla and Salkoff 1995; (5′ GGTGGAATATATACA...CAACCTTAAAAAATT 3′) was used to probe the same *Xba*I partial library, pBS-PAK2 was retrieved, purified, and sequenced. Sequenced portions of *PAK2* have only minor differences from the partial sequence of Jegla and Salkoff (1995).

PCR and RT-PCR were performed using Advantage cDNA polymerase mix (Clontech, Palo Alto, CA). A *Spe*I- and *Kpn*I-linker primer-pair specific for the *PAK1* open reading frame (ORF) (5′ oligo, GACTAGTATAAATGATACCCAAACTCCA AGGG; 3′ oligo, GGGGTACCTCATATAATTTTTTACACGAC TTATC), truncated *PAK1* Δ3′ (the same 5′ oligo and 3′ oligo: GGGGTACCTCATTGGTATGATAAGCAGCCA), or *PAK11* ORF (5′ oligos, GACTAGTATAAATGATACCCGGACTCTA AAAG and GACTAGTATAAATGATAACCCGGACTCTAAAA GATTAGAT for in-frame and frameshift, respectively; and 3′ oligo, GGGGTACCTCATATAATGTTTTATACCACTTATC for both; Operon, Alameda, CA) was used to amplify from total DNA or the first-strand cDNA. Additional primers up- and downstream of the ORFs had also been used in various RT-PCR experiments to finalize the boundaries of the ORFs. The first-strand cDNA was synthesized from purified 51s total RNA using oligo/poly(dT) with a SuperScript preamplification system (Life Technologies, Rockville, MD). All reactions were performed in a programmable thermal controller 100 (MJ Research, Watertown, MA). Reactions using genomic DNA as template yielded molecules of 2.4 kb. RT-PCRs yielded cDNAs, each with three introns spliced from their corresponding *PAK1* and *PAK11* genomic sequences, respectively.

The PCR-amplified genomic ORFs and RT-PCR-amplified cDNA ORFs of *PAK1* and *PAK11* were digested with the linker restriction enzymes and cloned into Paramecium expression vector pPXV at their corresponding sites (Haynes *et al.* 1995). At least two clones of each category were amplified and tagged for automatic sequencing using the ABI PRISM Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The reactions were then sequenced at UW Biotechnology Center (Madison, WI). Sequencing results were analyzed using EditView (Applied Biosystems) and SeqMan II (DNAStar, Madison, WI).

Preparation of plasmid DNAs for microinjection: Plasmids were digested with various restriction enzymes to completion as judged by gel electrophoresis. After digestion the plasmids were phenol-chloroform extracted, ethanol precipitated, and washed twice with 75% ethanol. All samples were resuspended in Tris-EDTA buffer pH 8.0 at 0.5–5 μg/μl for macronuclear injection. Standard molecular-biological techniques were used (Sambrook *et al.* 1989; Ausubel *et al.* 1999).

Microinjection for transgene expression in nd6 cells: An aliquot of 5–10 pl of the above-mentioned linearized plasmid at various concentrations was microinjected into the macronucleus of each recipient cell as previously described (Haynes *et al.* 1995). Recipients were cells from young clones of less than eight fissions after autogamy. Each plasmid sample was injected into six or more cells. The descendants of the individual recipients were cultured as individual clones and subjected to behavioral, electrophysiological, and molecular analyses.

Behavioral assay: Uninjected and microinjected cells were cultured for at least 24–48 hr before their behavior was tested. Cells were transferred into an adaptation solution [4 mm KCl, 1 mm $CaCl_2$, 1 mm *N*-(2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HEPES), 0.01 mm EDTA, pH 7.2] for 10 min to 1 hr. Then they were individually transferred into various testing solutions, including a Na^+-test solution (10 mm NaCl, 1 mm $CaCl_2$, 1 mm HEPES, 0.01 mm EDTA, pH 7.2), where the duration of continuous backward swimming of each cell was monitored using a stereomicroscope and a stopwatch (Kung *et al.* 1975).

Electrophysiology: Membrane currents were recorded from cells under voltage clamp using the established techniques (Preston *et al.* 1992). The intracellular capillary microelectrodes used for membrane potential recording and current injection contained 3 m KCl and had tip resistances of 20–40 MΩ. The cells were bathed in 1 mm KCl, 1 mm $CaCl_2$, 1 mm HEPES, and 0.01 mm EDTA, pH 7.2. Currents shown were filtered at 1 kHz and have been corrected for linear leak current as described (Preston *el al.* 1990a, 1992).

Analysis of total DNA by Southern blot and total RNA by Northern blot: Gel electrophoresis of total DNA isolated from descendant clones of uninjected nd6 controls and various microinjected transformants for Southern blots and their total RNA for Northern blots was performed as previously described (Haynes *et al.* 1995, 2000). The probes, *PAK11* ORF and calmodulin *CAM* ORF, were labeled using the *Redi*primeII random prime labeling system with [α-^{32}P]dCTP (Amersham Life Science, Arlington Heights, IL) as directed by the manufacturer. DNA marker was from GIBCO/Life Technologies (Alameda, CA) and RNA markers were from GIBCO/Life Technologies or Promega (Madison, WI). The radioactive signals were recorded on Phospho-Imager cassettes and then digitized and analyzed using ImageQuant 1.2 (Molecular Dynamics, Sunnyvale, CA).

Sequence comparison and secondary structure prediction: The protein sequence from the expected ORFs of *PAK1* and *PAK11* were used to do a homology search in the most recent databases employing several algorithms available at the National Center for Biotechnology Information web site. Secondary structures were analyzed using the PROTEAN program (DNAStar).

The sequence data presented in this article have been submitted to the EMBL/GenBank Data Libraries under accession nos. AF424539 (*PAK1*), AF432226 (*PAK2*), and AF424540 (*PAK11*).

RESULTS

The sequences for two very closely related K^+-channel genes, *PAK11* and *PAK1* (Ling *et al.* 1998), as well as a more distantly related *PAK2*, are germane to the present article (Jegla and Salkoff 1995; Ling *et al.* 1998). Corrections to the full-length sequences of *PAK11* and *PAK1*

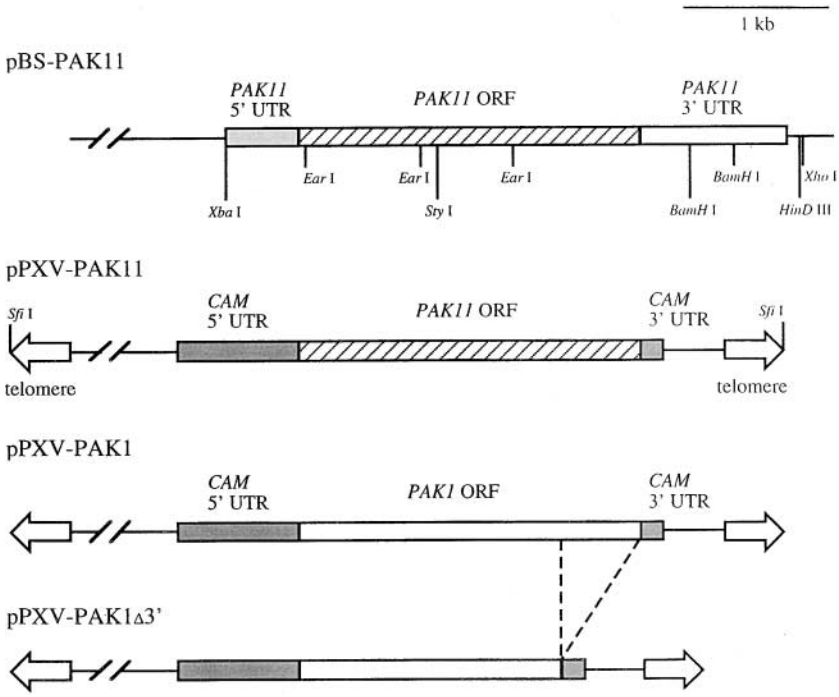

FIGURE 1.—Diagram of injected *PAK11* and *PAK1* plasmids. Solid lines represent the pBluescript or the pPXV vector sequence. Hatched bars represent the ORF of *PAK11* and open bars that of *PAK1*. Light-shaded bars represent the 5′ and 3′ UTRs of *PAK11* and dark-shaded bars that of *CAM*. Open arrows represent the telomeres.

were provided to GenBank. The ORFs of *PAK11* and *PAK1* were determined through successful RT-PCR amplification from the wild type using different gene-specific primer pairs. Within the very large superfamily of K^+ channels with six transmembrane α-helices, the *PAK*s studied here most closely resemble the AKT/EAG/ERG group of K^+ channels that have potential cyclic nucleotide-binding sites in their C-terminal cytoplasmic domains (ROBERTSON *et al.* 1996; GANETZKY *et al.* 1999).

***PAK11* transgenes cause hyperexcitable behaviors:** We transformed wild-type paramecia by microinjecting plasmids into individual macronuclei and examined the clonal descendants of the recipients (HAYNES *et al.* 2000). The insert in the injected plasmid (pBS-PAK11) contained the entire *PAK11* ORF as well as 0.5 kb of the 5′ and 1.5 kb of the 3′ untranslated region (UTR) flanked by the restriction sites *Xba*I and *Xho*I (Figure 1). The descendants of wild-type cells injected with an *Xba*I-*Xho*I-double digest of pBS-PAK11 invariably showed hyperexcitable behavior readily recognized even in the culture medium: they spontaneously swim backward for longer than tens of seconds in contrast to the occasional 1 sec or less for the uninjected controls. When tested with the Na^+-test solution (see MATERIALS AND METHODS), they invariably swim backward continuously for 1 min or longer, distinctly different from the transient avoiding reactions of ~1 sec observed in uninjected cells. Repeated experiments with intact *PAK11* consistently produce this effect in numerous separate injections. Usually this hyperexcitable phenotype was observed after the first post-injection fission. In cases where the first fission was delayed for a variety of reasons, the injected cells themselves showed hyperexcitability after ~12 hr. The effect has complete penetrance, having been observed in all descendant cells in each clone and at all clonal ages up to ~20 fissions, but was lost after autogamy. This hyperexcitable behavioral phenotype is striking but unexpected. K^+ channels normally pass outward currents that repolarize the membrane after depolarization. Therefore, the overexpression of K^+ channels is expected to terminate rather than to prolong membrane depolarization (excitation).

Intact *PAK11* ORF in the transgene is required for the effect: It is not necessary to release the *PAK11* fragment from the vector to effect the clonal behavioral transformation. The same behavioral effect was observed by injecting pBS-PAK11 linearized with *Bam*HI or *Hin*dIII, which left the ~3-kb vector sequence still attached to the uncut *PAK11* fragment (Figure 1). In contrast, pBS-PAK11 linearized with *Sty*I or *Ear*I, which have cut sites within the *PAK11* ORF (Figure 1), was completely ineffective in the phenotypic transformation.

Artificially promoted *PAK11* ORF is also effective, but frameshift *PAK11* ORF is not: The flanking sequences and UTRs of the *PAK11* ORF may be involved in causing the peculiar phenotypic change described above. To test this possibility and to prevent possible multimerization of the ORF in the recipient macronucleus, we subcloned the *PAK11* ORF into pPXV, a Paramecium expression plasmid with the constitutive calmodulin promoter (*CAM* 5′ UTR) and *CAM* 3′ UTR (HAYNES *et al.* 1995). The plasmid pPXV contains telomeric sequences and has been shown to not multimerize in the macronucleus (HAYNES *et al.* 1995; MCCORMICK-GRAHAM *et al.* 1997). Injection of empty pPXV, linearized with *Sfi*I, did not affect the behavior of paramecia

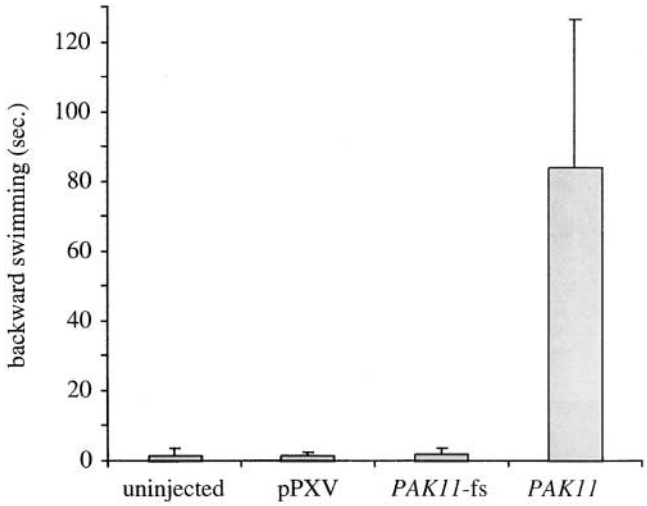

FIGURE 2.—Behavioral response of transgenic Paramecium. The descendants of wild-type cells injected with empty plasmid (pPXV), frameshift pPXV-PAK11 (*PAK11*-fs), or in-frame pPXV-PAK11 (*PAK11*) were compared to uninjected wild-type cells (uninjected). The duration of continued backward swimming upon transfer from an adaptation solution into the Na^+-test solution was monitored. Each value presented is the mean ± standard deviation of five cells from each of six separate clones.

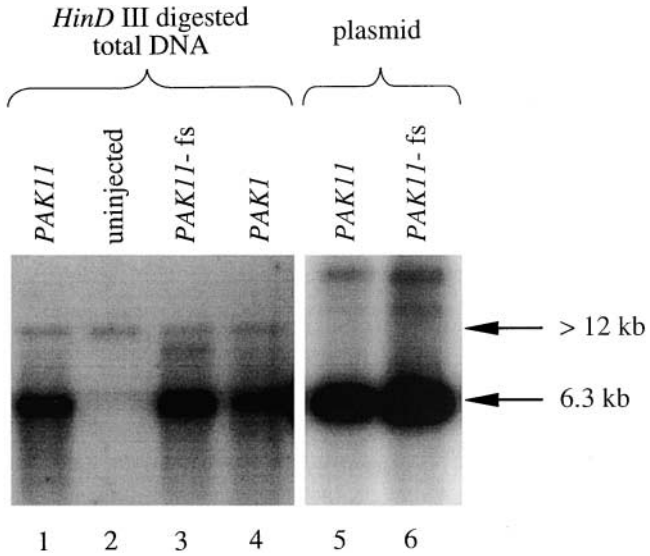

FIGURE 3.—Southern analysis of microinjected clones for *PAK11* endogene(s) and transgenes. Total DNAs extracted from various clones were digested with *Hin*dIII before being electrophoresed in a 0.7% agarose gel at ~1 μg per lane alongside lanes loaded with *Sfi*I-digested plasmids used in the injection experiment. The plasmids have no *Hin*dIII site. The blot was probed with random-primed ^{32}P-labeled *PAK11* ORF. Clones from cells injected with pPXV-PAK11 (lane 1), frameshifted pPXV-PAK11 (lane 3), and pPXV-PAK1 (lane 4) all have the same prominent 6.3-kb band present in the *Sfi*I-digested pPXV-PAK11 (lane 5) and frameshifted pPXV-PAK11 (lane 6), indicating the transgenes have replicated in the descendants. All clones, including the uninjected control (lane 2), have a faint band of >12 kb.

(Figure 2). On the other hand, *Sfi*I-linearized pPXV-PAK11 induces the same hyperexcitable behavior in the clonal descendants of the injected cells as observed above (Figure 2). Molecular evidence verifies that the injected plasmids indeed replicated autonomously in the transformed macronuclei with little multimerization (see below).

A shift in the reading frame should allow us to discern whether it is the nucleotide sequence in the PAK11 RNA or the amino acid sequence of the translated PAK11 protein that causes the hyperexcitable phenotype. Accordingly, we engineered a pPXV plasmid with a single base-pair insertion downstream of the start codon of the *PAK11* ORF, frameshifted (fs) pPXV-PAK11, and tested for its transformation effect. This insertion (5′ ATGATAC . . . to 5′ ATGATA<u>A</u>C . . .) was expected to shift the reading frame and to terminate the translation at amino acid residue 32. As shown in Figure 2, this plasmid, which was also linearized with *Sfi*I, is entirely ineffective in producing hyperexcitability.

***PAK2* does not have the same effect as *PAK11* and *PAK1*:** While *PAK1* and *PAK11* are 96% identical, *PAK2* and *PAK1* have only 24% identity between their transmembrane amino acid sequences (JEGLA and SALKOFF 1995) except in the regions that are suggested to encode the channel K^+ filter where sequence identity is high. We constructed pBS-PAK2 with a 4.5-kb insert encompassing the *PAK2* ORF and its UTRs. Injection of *Bam*HI-digested pBS-PAK2 did not generate any hyperexcitable clones. We subcloned the entire genomic *PAK1* ORF into pPXV to form pPXV-PAK1. *Sfi*I-linearized pPXV-PAK1 produced hyperexcitable clones indistinguishable from those transformed by *Sfi*I-linearized pPXV-PAK11 (Figure 2). We also constructed a pPXV bearing a truncated version of *PAK1* ORF, pPXV-PAK1 Δ3′ (Figure 1), that encodes all of the six transmembrane domains, including the filter and the pore, as well as the first 179 amino acids of the cytoplasmic C-terminal end followed by a TGA stop. Injection of a *Sfi*I digest of this construct was just as ineffective as the *Sfi*I digest of frameshifted pPXV-PAK11 described above (data not shown).

Fate of the injected transgenes in *PAK11* and *PAK1*: Our previous work demonstrated that the linearized pPXV expression plasmid and its insert consistently replicate faithfully and autonomously in Paramecium macronuclei (HAYNES *et al.* 1995; HAYNES *et al.* 1998). Here, the molecular status of the *PAK* transgenes and the endogenes in the transformed clones was also examined. The entire *PAK11* ORF was used to probe a complete *Hin*dIII digest of total DNA extracted from the various clones. In all the phenotypically transformed clones, the major signal was at 6.3 kb (Figure 3, lanes 1, 3, and 4), matching exactly the size of the *Sfi*I-cut in-frame and frameshifted pPXV-PAK11 plasmids originally used for injection (Figure 3, lanes 5 and 6). Within the resolution of the blot, there were no signs of dele-

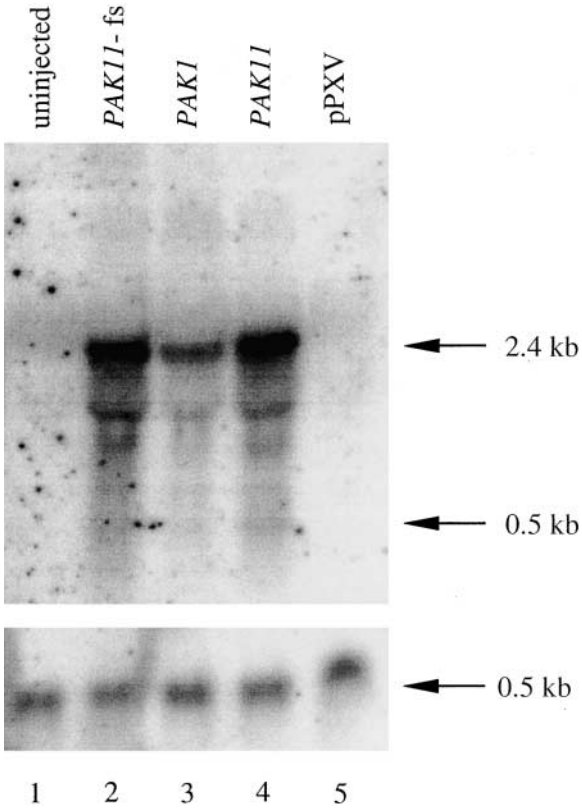

FIGURE 4.—Northern analysis of microinjected clones. Total RNAs purified from various clones were loaded at ~25–30 μg/lane and separated in a 1.5% formaldehyde-agarose gel. The blot was first probed with random-primed ^{32}P-labeled *PAK11* ORF (top) and then reprobed with random-primed ^{32}P-labeled *CAM* ORF (bottom). Clones descended from cells injected with frameshifted pPXV-PAK11 (lane 2), in-frame pPXV-PAK1 (lane 3), and pPXV-PAK11 (lane 4) have a major band of 2.4 kb, which is not detectable in the uninjected clone (lane 1) and pPXV-injected control clone (lane 5). But all clones have a calmodulin message band of 0.5 kb.

tion, insertion, or multimerization, indicating faithful autonomous replication of the linearized plasmid over the many fissions after injection. The 6.3-kb major signal is absent in DNA from uninjected clones (Figure 3, lane 2). All DNA samples have a band >12 kb in size, assumed to be the result of hybridization to the genomic DNA. This >12-kb band may represent multiple endogenes since the PAK11 probe apparently can detect both *PAK1* and *PAK11* sequences (see below).

***PAK11* trangenes are transcribed and translated:** We also used the entire *PAK11* ORF to probe total RNAs from the various descendant clones. A 2.4-kb hybridization signal is evident in the clones transformed with *Sfi*I-linearized pPXV-PAK1 or pPXV-PAK11 that show the hyperexcitable phenotype (Figure 4, lanes 3 and 4, top). The same 2.4-kb Northern signal is present in the clones from cells injected with the pPXV bearing the frameshifted *PAK11* ORF, which, as stated above, shows no hyperexcitability (Figure 4, lane 2, top). These signals match the expected size of the *PAK1* and *PAK11* RNA and indicate that the transgenes are being transcribed whether they are cloned in-frame or off-frame. Taking into consideration the loading control (Figure 4, bottom) and the Southern signals of *PAK* transgenes (Figure 3), the failure of the frameshifted pPXV-PAK11 to transform was clearly not because of underinjection. Clones transformed with an empty pPXV (Figure 4, lane 5, top) and those of uninjected cells (Figure 4, lane 1, top), which do not show any phenotypic changes, have no recognizable signal, 2.4-kb or otherwise. Thus, the transcripts from the endogenes are far fewer compared to those from the large number of transgenes.

***PAK11* transformants have reduced K^+ currents:** While swimming behaviors reflect the activities of multiple ion channels and pumps, currents through individual channels can be identified by their charge carrier, kinetics, and other biophysical characteristics. To determine which of the previously described activities might be responsible for the hyperexcitable phenotype of the pPXV-PAK11-transformed wild-type paramecia, we examined individual ion currents using a two-electrode voltage-clamp analysis. Membrane currents were examined under conditions optimal for Ca^{2+} and K^+ conductance (see MATERIALS AND METHODS). Four principal K^+ currents have been described to date (SAIMI and KUNG 1987; PRESTON *et al.* 1990a). The first two are activated upon membrane depolarization and were elicited using 1500-msec steps from rest (−40 mV) to potentials between −35 mV and 0 mV. Examples of currents elicited by a step from −40 mV to −5 mV are shown in Figure 5, left. The voltage step (V_m) first elicited a rapid inward Ca^{2+} transient (asterisk in upper current trace in Figure 5), followed by an outward, voltage-gated K^+ current ($I_{K(d)}$). The magnitude of this delayed rectifier could be approximated from the peak current at 50–100 msec (Table 1). A second, Ca^{2+}-dependent K^+ current ($I_{K(Ca,d)}$) developed slowly during step depolarizations toward a maximum at 1–1.5 sec (Figure 5, single arrow) and yielded a slowly decaying tail current upon returning to −40 mV (Figure 5, double arrows, and Table 1). We found no significant difference in the amplitude of $I_{K(d)}$ between uninjected control cells and clonal descendants of cells injected with frameshift and in-frame PAK11 plasmids (Table 1). We did record a significant decrease in $I_{K(Ca,d)}$ in pPXV-PAK11 transformants, however, as reflected in tail current amplitude at 20 msec (Table 1). This finding is consistent with the behavioral observation suggesting that the hyperexcitable phenotype results from suppression of the same K^+ current that is affected in pantophobiac mutants. We also examined the two K^+ currents that are activated specifically upon hyperpolarization. The currents shown in Figure 5 (right) were elicited by 500-msec steps to −120 mV from rest. The inward peak (asterisk) consisted largely of a hyperpolarization-induced Ca^{2+} current and a voltage-gated K^+ current ($I_{K(h)}$). A Ca^{2+}-dependent K^+ current ($I_{K(Ca,h)}$) activated more slowly during the voltage

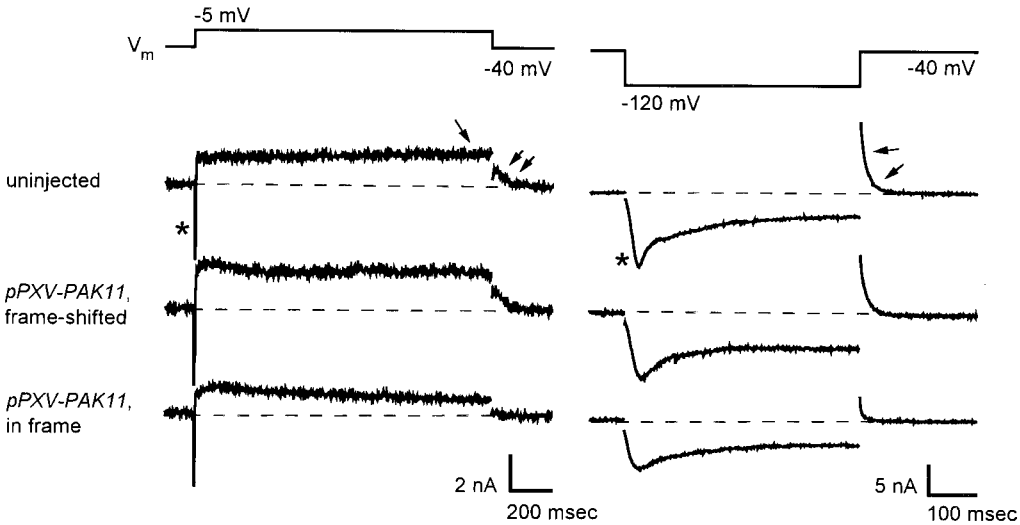

FIGURE 5.—Ion currents in control and injected cells. Membrane currents were elicited under two-electrode voltage clamp from cells bathed in 1 mM K^+. The top indicates the step changes in membrane potential (V_m) used to elicit the currents shown below. Traces at left were elicited using 1.5-sec steps from −40 to −5 mV. In uninjected control cells, this elicits a rapid inward Ca^{2+} transient (asterisk) followed within a millisecond by a voltage-dependent K^+ current. A Ca^{2+}-dependent K^+ current develops more slowly during the step toward a maximum at 1.5 sec (arrow) and manifests as a prominent outward tail current that decays slowly upon a return to rest (double arrows). A similar sequence of currents is observed upon step depolarization of clonal descendants of cells injected with frameshifted *pPXV-PAK11*, but in-frame transformants lack the slow Ca^{2+}-dependent K^+ component noted in the controls. Note that this manifests both as a reduced outward current during the step and as loss of the corresponding outward tail. Traces at right were elicited using 500-msec steps to −120 mV. This elicits an inward peak (asterisk) that consists of both a hyperpolarization-activated Ca^{2+} current and a second voltage-dependent K^+ current. A Ca^{2+}-dependent K^+ current develops over a period of 50–400 msec during step hyperpolarization. The outward tail current elicited upon returning to −40 mV decays biexponentially, reflecting the coincident decay of the two K^+ currents (indicated by the two arrows). Both tail components are apparent in frameshifted *pPXV-PAK11* transformants but descendants of in-frame transformants lack the Ca^{2+}-dependent currents.

step and was the principal contributor to the late inward current at 500 msec. The tail current elicited upon returning to −40 mV (Figure 5, double arrows) decayed biexponentially, reflecting the time course of deactivation of the two K^+ currents. By fitting these tails to two exponentials, it was possible to determine the individual contributions of the two K^+ currents to the total current; their amplitudes are given in Table 1. Again, we found no significant difference in amplitude of the voltage-gated K^+ current among the three populations of cells, but the Ca^{2+}-dependent current was significantly reduced by transformation with pPXV-PAK11.

DISCUSSION

Transgene-induced loss of K^+ currents is not a common "gene silencing" phenomenon: One common strategy to study eukaryotic channels is to express them in Xenopus oocytes, which can be readily manipulated and examined using electrophysiological and other experimental techniques. Since Paramecium uses UAA and UAG to encode glutamines (PREER *et al.* 1985) and *PAK1* or *PAK11* each has 21 such codons, they needed to be changed before they could be expressed heterologously. To investigate their corresponding currents, we therefore expressed them as transgenes in Paramecium itself. We might have expected that overexpression of the *PAK* genes would enhance cell permeability to K^+, but instead we observed a behavior reminiscent of the pantophobiac mutants that are characterized by their deficits in Ca^{2+}-activated K^+ currents (HINRICHSEN *et al.* 1986; PRESTON *et al.* 1990b). Pantophobiac mutants were first isolated for their hyperexcitable behavior that correlated with the loss or reduction of the Ca^{2+}-dependent

TABLE 1

Comparison of K^+ currents

		PAK11	
K^+ current (nA)	Uninjected	Frameshifted	In-frame
$I_{K(d)}$	2.84 ± 0.87 (3)	4.49 ± 1.49 (3)	2.67 ± 1.74 (5)
$I_{K(Ca,d)}$	1.15 ± 0.33 (4)	1.35 ± 1.03 (3)	0.11 ± 0.15 (5)*
$I_{K(h)}$	3.21 ± 1.24 (3)	5.87 ± 0.81 (3)	3.83 ± 1.72 (5)
$I_{K(Ca,h)}$	14.14 ± 9.68 (4)	15.6 ± 5.04 (3)	0.92 ± 1.09 (5)*

The two currents activated upon depolarization were elicited using 1500-msec steps from −40 to −5 mV, the currents upon hyperpolarization by 500-msec steps to –120 mV. See text on current measurements. Mean ± SD(n), where n is the sample size. Asterisks indicate a significant difference between these results and both uninjected and injected controls, $P < 0.05$, t-test.

K^+ current activated upon depolarization (Saimi *et al.* 1983) as well as that upon hyperpolarization (Preston *et al.* 1990a). The mutations were eventually traced to the calmodulin gene (Kink *et al.* 1990; Saimi and Kung 1994). Direct measurements of the various membrane currents under voltage clamp confirmed that the Ca^{2+}-dependent K^+ currents were indeed attenuated in the *PAK11* transformants (Figure 5 and Table 1). The phenomenon is robust, as *PAK1* or *PAK11* transgene invariably causes the dramatic hyperexcitable behavior (Figure 2) in the descendant clones that persists until the next autogamy, a process in which the transgene-bearing macronuclei are replaced by new macronuclei derived from the germ nucleus. At this level of phenotypic observation, the phenomenon resembles most cases of gene silencing in that the transgene leads to a loss of the activity of the gene product, such as the cases in Paramecium reported by Ruiz *et al.* (1998). In most cases of sequence-specific gene silencing in other species currently under study, the loss of the activity is due to a reduction in the targeted mRNA (see Baulcombe 2000; Cogoni and Macino 2000; Sharp 2001 for reviews.) In the present studies, transgene-derived RNA was easily detected although the RNA from the endogene is below the level of detection of our hybridization (Figure 4). The fact that we can clone intron-free cDNAs of *PAK11* and *PAK1* endogenes by RT-PCR from uninjected wild-type cells demonstrates the presence of both specific RNA templates in Paramecium. In addition, clonal hyperexcitability is inducible by pBS-PAK11. In it, the *PAK11* transgene is identical to the endogene flanked by ~2 kb of flanking UTR sequences. We therefore reason that the *PAK11* endogene is also transcribed in the hyperexcitable transformants. Preliminary investigation with pPXV-PAK11-green fluorescent protein fusion constructs elicited both clonal hyperexcitability and a green fluorescent signal that are absent in the uninjected controls. While this indicates that the transgene message was actually translated, further study is underway to characterize the nature of this translated product.

The effects are likely due to the channel protein: A single nucleotide insertion at the 5′ end of the ORF should not greatly affect the structure and function of RNA. On the other hand, this insertion would shift the reading frame and abolish translation. Our frameshifted pPXV-PAK11 construct produces RNA of the same size as the in-frame plasmid (Figure 4). The fact that it is entirely ineffective in producing the hyperexcitable phenotype (Figure 2) supports the hypothesis that the effect is not due simply to the presence of transgenic *PAK11* RNA but to the production of the protein. The possibility that the frameshifted RNA is somehow specifically destroyed is refuted because the level of plasmid-derived *PAK11*-specific RNA is approximately the same in both cases (Figure 4). We also found that the *PAK* ORF in the plasmids needs to be intact for the hyperexcitability to occur: truncating or bisecting the ORF invariably removes the phenotypic transformation. Furthermore, the effect does not depend on the specificity of the untranslated portions of the transgenic *PAK* gene since the ORF placed behind its own promoter and 3′ UTR or the *CAM* promoter and 3′ UTR have the same effect. These results indicate that the hyperexcitability is the consequence of the translational product of the *PAK11* transgenes in the macronucleus. The failure of the truncated *PAK1* transgenes to transform indicate that the C-terminal region of this K^+-channel subunit could be critical in assembly and/or functioning. Direct localization and quantification of PAK11 proteins await further investigation with fusion constructs and antibodies.

Meanwhile, we have used the specific electric currents through these channel proteins to follow their individual activities. Because each type of channel has its own ion permeability, gating parameters, activation and inactivation kinetics, as well as its own set of specific blockers, each can be studied separately under a voltage clamp *in vivo* (Hille 1992). Our previous investigations have shown the loss of two types of Ca^{2+}-dependent K^+ currents in the pantophobiac mutants (Preston *et al.* 1990b). The present studies have suggested that the presence of either *PAK1* or *PAK11* transgenes yield pantophobiac-like hyperexcitability by suppressing these same Ca^{2+}-dependent currents (Figure 5). Since both $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$ require an increase in the intracellular free Ca^{2+} concentration to be activated, we have to consider the possibility that overexpression of the *PAK* genes attenuates these currents through suppression of the Ca^{2+} currents activated upon depolarization and hyperpolarization, respectively. This is clearly not a plausible explanation for the phenotype, because suppressed Ca^{2+} currents would have decreased the duration of backward swimming and inward Ca^{2+} currents are clearly observed upon both step depolarization and hyperpolarization (asterisks in Figure 5). A complete inventory of all known membrane currents in the various transformant clones is far beyond the scope of the present work. Therefore, we cannot be certain that currents other than the four K^+ currents listed in Table 1 are not also affected by *PAK1* or *PAK11* injection. We have noted variations in the amplitudes of several currents between control and injected cells, but they may prove to be statistically insignificant upon further scrutiny. Regardless, they are unlikely to be the cause of the obvious deficit in $I_{K(Ca,d)}$ and $I_{K(Ca,h)}$ (Table 1) and the hyperexcitable behavioral phenotype described here.

Possible mechanism(s): There are now over 15 known ORFs, each capable of specifying one type of K^+-channel subunit, at least conceptually, in Paramecium. If we assume that Paramecium K^+ channels are heterotetramers, and each type of channel has a different subunit composition, the overabundance of one type of subunit from the expression of a certain *PAK* transgene may disrupt tetramer assembly. Misassembled nonfunctional tetramers may compete with the functional ones and perturb channel production, secretion, and deployment into limited surface sites. In addition, nonfunctional

proteins may trigger degradation that removes even the functional proteins. The observation that the two Ca^{2+}-dependent K^+ channels are strongly affected by the *PAK1* or *PAK11* transgenes but not by *PAK2* indicates that these two channels are most sensitive to ill effects of overproduction of PAK1-like subunits. It seems possible that these two channels normally use PAK1 subfamily members as some or all of their subunits and the natural proclivity of accepting these subunits into the tetramers is being abused here by the excessive abundance of one type of subunit produced by the transgene. In Drosophila, both the *Sh* (*shaker*) and *Eag* (*ether-a-go-go*) loci encode different K^+-channel subunits. Wu *et al.* (1983) and Zhong and Wu (1991) showed that, in larval muscles, mutant *eag* produces multiple types of K^+ currents and different alleles have quantitatively different effects. Coexpression of *Sh* and *Eag* in oocytes produces K^+ currents that are different from the expression of *Sh* alone, and the interaction among the different channel subunits depends on both the amount of RNA injected and the length of time after the injection (Chen *et al.* 2000). These results were interpreted as the effects of coassembly of different subunits into the K^+-channel tetramers. Among the superfamily of cation channels with six transmembrane α-helices, *PAK*s are most similar to the *Eag* group. It therefore seems possible that they also enter heterotetrameric assembly. The C-terminal cytoplasmic domain of the *Eag* subunit has a cyclic nucleotide-binding site (Guy *et al.* 1991) and the *Eag* current expressed in oocytes is modulated by cAMP (Bruggemann *et al.* 1993). One of the *PAK*-like genes revealed in the Paramecium genome sequencing project (Dessen *et al.* 2001) apparently encodes a polypeptide, a portion of which has all the canonical features of the same cyclic nucleotide-binding domain. PAK1 and PAK11 polypeptides seem to have a similar domain but also have substitutions of two conserved residues in the nucleotide (McCue *et al.* 2000). These changes are not likely artifacts of our DNA manipulations, since they are identical in PAK1 and PAK11 and in multiple PAK11 sequences derived from different Paramecium stocks. The significance of these changes is unknown, but may be related to the suppression-of-K^+-currents effect discussed here.

Gene silencing and reverse genetics in Paramecium: In most cases of sequence-specific gene silencing currently under study in other species, the loss of the activity is due to a reduction in the targeted mRNA. This occurs either by reducing the amount of the gene being transcribed (transcriptional silencing) or by degrading the RNAs produced (post-transciptional silencing; Baulcombe 2000; Cogoni and Macino 2000; Sharp 2001). Ruiz *et al.* (1998) showed clearly that transgenes reduced gene expression in a sequence-specific manner by a post-transcriptional mechanism in Paramecium. They showed that this phenomenon can effect the expression of both unique genes as well as members in gene families. The discovery of a silencing phenomenon in Paramecium is an important development. Combined with the sequence information yielded by the ongoing Paramecium genome project (Dessen *et al.* 2001), the physiological functions of individual gene products can be investigated *in vivo*. This article shows that the actual mechanism for a particular loss-of-function phenotype, induced by injection of plasmids, may not always be a reduction of transcripts. Our observation of the reduction of two separate Ca^{2+}-dependent K^+ currents upon the expression of *PAK11* or *PAK1* is apparently a consequence of a post-translational rather than a post-transcriptional mechanism. The results of the present work do not, however, preclude the possibility of inducing post-transcriptional silencing of the *PAK* genes using a different protocol. Ruiz *et al.* (1998) not only examined a different set of genes, but also used a different protocol. During our initial attempts to characterize the physiological role of the many homologs to metazoan K^+ and Na^+/Ca^{2+} channels uncovered in the Paramecium genome project, silencing will be a valuable technique. At the same time we must improve our understanding of the diversity of mechanisms to allow for more specific and effective investigations of any one particular gene.

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