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Identification and structural characterization of a cDNA clone encoding a membrane-bound form of the polypeptide pheromone Er-1 in the ciliate protozoan *Euplotes raikovi*

(cell recognition signals/gene structure and expression/mating types)

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ABSTRACT **In the ciliate *Euplotes raikovi*, the same cell that secretes the pheromone Er-1, a polypeptide of 40 amino acids derived from a precursor (prepro-Er-1) of 75 amino acids, also produces a polypeptide of 130 amino acids, of which the 75 residues at the carboxyl terminus are identical to those of prepro-Er-1 and the 55 residues at the amino terminus form a new sequence. This larger Er-1 isoform is retained in membranes, where it may function as a binding site for soluble Er-1 in a mechanism of autocrine secretion. Membrane-bound and soluble Er-1 are translated from two mRNAs that apparently originate from a common micronuclear and/or macronuclear gene through alternative elimination of intervening sequences. This finding suggests that single genes responsible for the generation of isoform diversity in polypeptide hormones are present even in single-celled eukaryotes.**

Families of polypeptide factors (designated mating pheromones or, simply, pheromones) are specified by the highly polymorphic genetic locus *mat* (1) and are constitutively secreted into the extracellular environment by some species of *Euplotes* (2), a genus of commonly occurring and evolutionarily advanced protozoan ciliates. Each pheromone confers a distinctive molecular phenotype (or mating type) on the cell of origin and may induce other cells to transform for combining with each other in mating pairs. Accordingly, ciliate pheromones have been functionally compared to sex factors and thus proposed to specifically control reciprocal cell–cell recognition and induction of mating activity (3, 4). However, there is evidence that they function in an autocrine fashion by binding to high-affinity receptors on the same cell that secretes them, thereby primarily regulating the vegetative cell life (2, 5).

Among the pheromones of *Euplotes raikovi* purified to date (6–8), each one distinctive of one cell type and controlled by one of a series of alleles codominantly expressed at the *mat* locus (9), pheromones Er-1, Er-2, and Er-10 have been structurally characterized by both amino acid and nucleotide sequence analyses (refs. 8, 10, 11, and 38; unpublished data). Each is synthesized as a precursor of 75 amino acids (prepropheromone) and subsequently is processed to the mature form (11, 34). The secreted, native pheromones are composed of 38–40 amino acids with three intrachain disulfide bridges and occur as oligomers, apparently as homodimers or some higher-order aggregates (10, 12).

In this report, we show that type I cells, which secrete Er-1 and are homozygous for the allele *mat-1*, also produce a membrane-bound form of Er-1 and that these two isoforms are encoded by two distinct mRNAs that appear to be produced through a mechanism involving an alternative excision of intervening sequences (IVSs).‡

MATERIALS AND METHODS

cDNA Library Construction and Screening. The preparation of the *E. raikovi* cDNA library in a pUC12 vector has been described (11). The library screening to select clone p5/6 was carried out by colony hybridization, using clone p45/2 as a probe labeled by random priming (13). Colony hybridization was performed according to Sambrook *et al.* (14), with a temperature of 65°C and a washing solution of 0.15 M NaCl/0.015 M sodium citrate, pH 7.

Oligonucleotide Synthesis. An Applied Biosystems model 308A DNA synthesizer, with procedures and reagents recommended by the manufacturer, was used for the synthesis of the following three oligonucleotides corresponding to the sequence stretches indicated in Fig. 2*A*: 5′-AGCCTATAATGTCATAGAATG-3′, 3′-TGTAGATAAGATTGACGGGTGGAATACAG-5′, 3′-AATAACTTGTTTCAAGGTAGA-5′.

Reverse Transcription–Polymerase Chain Reaction (RT–PCR). Poly(A)$^+$ RNA (2 μg) from type I cells was pretreated with 10 units of RNase-free DNase I (Bethesda Research Laboratories), to avoid any possible DNA contamination, in the presence of 40 units of RNase inhibitor (Bethesda Research Laboratories) and 2 mM $MgCl_2$ for 1 hr at 37°C. The reaction mixture was heated at 95°C for 5 min to inactivate DNase I and immediately cooled on ice. DNase-treated RNA was incubated with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) for cDNA synthesis, according to the conditions suggested by the enzyme supplier. The sample was phenol-extracted, ethanol-precipitated, and centrifuged to recover the cDNA pellet. Half of the resuspended pellet was used as template for the amplification carried out according to standard Cetus conditions (15), with 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. A final incubation at 72°C for 7 min was added to the last cycle.

PCR. PCR was performed under the same conditions as reported above but using as template 0.5-μg samples of DNA extracted from macronuclei purified from type I cells (11).

DNA Sequencing. For the sequence determination of clone p5/6, the insert was excised from pUC12 vector by *Bam*HI and *Sst* I restriction enzymes (Boehringer Mannheim) and digested as shown in the restriction map of Fig. 1. The fragments were subcloned into M13mp8 (or M13mp9) and pUC13 vectors. Sequencing was performed by the dideoxy

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Abbreviations: IVS, intervening sequence; RT–PCR, reverse transcription–polymerase chain reaction.

‡The sequence reported in this paper has been deposited in the GenBank/EMBL data base (accession no. M86864).

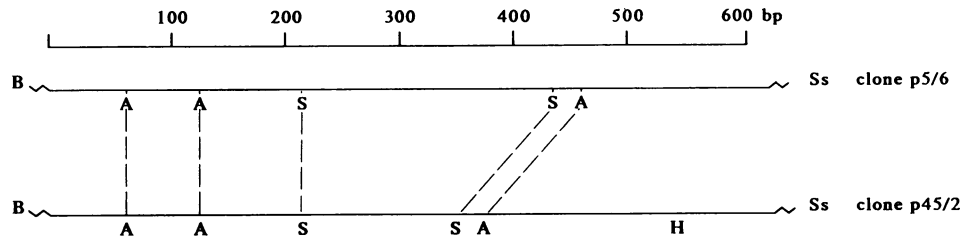

FIG. 1. Restriction maps of clones p5/6 and p45/2. Nucleotide scale is indicated in base pairs (bp). Wavy lines indicate the pUC12 vector regions flanking the inserts. DNA was digested with enzymes *Alu* I (A), *Sau*3A1 (S), *Bam*HI (B), *Hin*cII (H), and *Sst* I (Ss). The restriction sites indicated were also used for subcloning and sequencing.

chain-termination method (16, 17) with Sequenase (United States Biochemical) and pUC sequencing (Boehringer Mannheim) kits. For sequencing of DNA amplified by PCR, each amplified product was purified by electrophoresis in low-melting agarose gel and the sequencing primer was end-labeled by phosphorylation of the 5′ hydroxyl with [γ-^{32}P]ATP in a reaction using T4 polynucleotide kinase (11). The primer (5–10 pmol) was annealed to half or all of the amplified product for direct sequencing by the dideoxy method (16) with the modifications suggested by Murray (18).

Preparation of Er-1 Antibodies. Er-1 antibodies were prepared by immunizing a New Zealand White rabbit with pure Er-1 samples (6). For the first immunization, a 1-mg sample dissolved in physiological solution was mixed with an equal volume of Freund's incomplete adjuvant and injected subcutaneously into the rabbit. Three booster injections (1 mg every 2 weeks) were given. The rabbit was bled after every immunization and immunoglobulins were precipitated by 40% saturated ammonium sulfate. The Er-1 antiserum titer was determined by ELISAs before the immunoglobulins were used.

Immunoblot Analyses. Membrane proteins and total cell extracts were obtained from type I cells of cultures in early stationary phase of growth. Membrane proteins were prepared from cell cortex pellets and solubilized with octyl β-D-glucopyranoside (5). Total cell extracts were prepared by sonication and suspension with 0.1 M $NaHCO_3$/0.5 mM EDTA/1 mM phenylmethylsulfonyl fluoride/0.5% SDS, at pH 7.2. Preparations were boiled in SDS sample buffer with 5% (vol/vol) 2-mercaptoethanol and fractionated by electrophoresis (19) in a 10–20% polyacrylamide gradient gel run with two mixtures of standard molecular weight markers, one in the range of 92,000–14,000 (Bio-Rad) and the other in the range of 29,000–6500 (Serva). Proteins were transferred to nitrocellulose or poly(vinylidene difluoride) (Immobilon, Millipore) filters in a Trans-Blot apparatus (Bio-Rad). Transfer conditions for nitrocellulose filters were 6 hr, 70 V, 4°C, and a buffer containing 25 mM Tris, 192 mM glycine (pH 8.3), and 10% methanol; for transfer to Immobilon filters the manufacturer's suggestions were followed. Filters were then rinsed in Tris-buffered saline (TBS: 20 mM Tris·HCl, pH 7.5/500 mM NaCl) and incubated for 3 hr with 3% bovine serum albumin in TBS to block nonspecific protein-binding sites. Blocked filters were incubated overnight with Er-1 antibodies diluted 1:200 in 1.5% albumin in TBS. Subsequently, filters were washed in TBS containing 0.05% Tween 20 and incubated for 2 hr at room temperature with swine anti-rabbit immunoglobulins (Dakopatts, Glostrup, Denmark) diluted 1:800 in TBS containing 0.05% Tween 20 and 1.5% albumin. To enhance the amount of peroxidase for the staining reaction, filters were incubated with peroxidase–anti-peroxidase complexes (Dakopatts) diluted 1:500 in TBS containing 0.05% Tween 20 and 1.5% albumin. After two further washes with TBS containing 0.05% Tween 20, filters were stained with 4-chloro-1-naphthol (5 mM in methanol) in the presence of 0.03% H_2O_2.

RESULTS AND DISCUSSION

DNA Sequence Determination of Clone p5/6. The nucleotide sequence of the prepro form of the secreted pheromone Er-1 was determined previously (11) from analyses of two overlapping cDNA clones isolated from cDNA libraries in the vectors λgt10 and pUC12. More recently, another cDNA clone, p5/6, has been identified in the same pUC12 library by cross-hybridization with a full-length Er-1 cDNA clone, p45/2, that was obtained from a new, restricted library constructed from RNA enriched in Er-1 mRNA. As shown in Fig. 1, the restriction map of clone p5/6 is identical to that of clone p45/2, except for two regions distinguished by a longer *Sau*3A1 fragment and the lack of a *Hin*cII restriction site. As revealed by DNA sequence analyses (Fig. 2), these two regions of clone p5/6 are characterized by an insertion and a deletion, respectively. The former, designated 5′[p5/6] IVS, consists of a sequence of 89 nucleotides and is located in the 5′ half of the molecule; the latter, denoted 3′[p45/2] IVS, is defined by a sequence of 78 nucleotides that, in clone p45/2, is inserted close to the polyadenylylation signal of the 3′ noncoding region. Because of the insertion, a longer open reading frame appears in clone p5/6 with respect to that of clone p45/2. One ATG codon commencing at nucleotide 183 and another at nucleotide 210 appear in frame with the ATG codon at position 348, which initiates translation in clone p45/2. Of the codons at 183 and 210, the former is obviously a better candidate to function as an initiation site in clone p5/6 since, as with most eukaryotic mRNA sequences, it shows a purine at nucleotide −3 and is located immediately downstream from the sequence TAGA, which similarly precedes the first translated ATG (at position 348) in clone p45/2. It is less likely that the ATG codon at 210 functions as an initiation codon since it is preceded by a sequence that is completely different from the CCA/GCCATG consensus sequence proposed to function as a translation initiation site in eukaryotes (20). If the ATG codon at 183 is the initiation site utilized in the expression of the transcript corresponding to clone p5/6, the product of this gene is a polypeptide of 130 amino acids. Of these, the carboxyl-terminal 75 amino acids are identical to prepro-Er-1, which arises from the initiation site at nucleotides 348–350 (11). The amino-terminal 55 residues constitute a new sequence.

Evidence of Two mRNAs. Due to a difference in length of only 11 bases, the mRNAs of clones p5/6 and p45/2 comigrate as a single band of about 680 bases in RNA blotting analyses. To distinguish these two mRNAs, the RT–PCR procedure was used. cDNA molecules were produced by reverse transcription of poly(A)$^+$ RNA of type I cells carried out with the 29-mer oligonucleotide corresponding to the sequence localized (as shown in Fig. 2*A*) 229 nucleotides downstream of the 5′[p5/6] IVS; this same oligonucleotide was then used for the amplification reaction together with a 21-mer oligonucleotide corresponding to the sequence beginning 61 nucleotides upstream of the same IVS. Two amplified products of the expected lengths, 319 and 408 bp, were obtained; the difference of 89 bp corresponds to the 5′[p5/6]

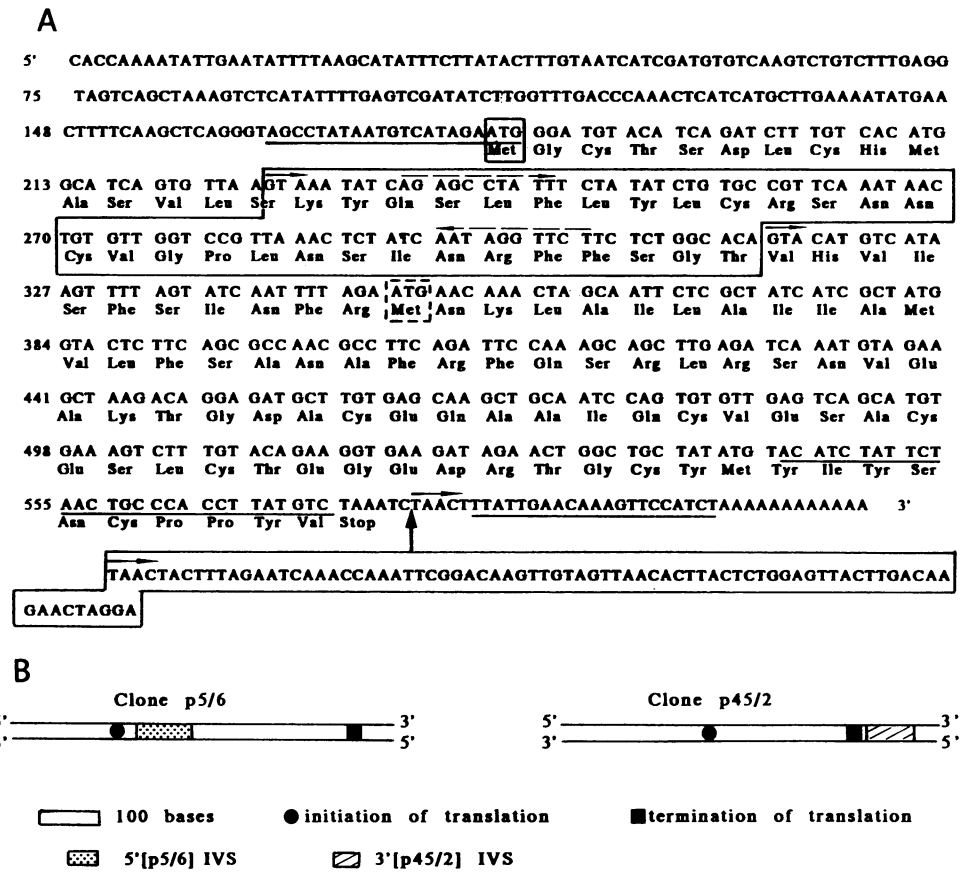

FIG. 2. (*A*) Nucleotide sequence of clone p5/6 showing the predicted amino acid sequence. The 5'[p5/6] and 3'[p45/2] IVSs are boxed and the insertion site of the latter is indicated. Solid arrows indicate short direct repeats at the junctions of the two IVSs, and dashed arrows indicate internal inverted repeats. Sequences corresponding to oligonucleotides used as primers for PCR are underlined. The ATG codon proposed to initiate translation is indicated by a solid box, whereas a dashed box indicates that initiating translation in clone p45/2. (*B*) Schematic diagrams of clones p5/6 and p45/2 showing the IVS position with respect to initiation and termination of translation sites.

IVS deletion in clone p45/2. As shown in Fig. 3, these products were stained by ethidium bromide in an agarose gel and appeared as two bands of appreciably different intensities (most likely reflecting different amounts of the two transcripts) in a Southern blot analysis after hybridization with the insert of clone p5/6.

Cell Localization of the Product of Clone p5/6. A consequence of incorporating the full sequence of the exported pheromone precursor (prepro-E*r*-1) into the larger molecule encoded by clone p5/6 is to potentially convert the amino-terminal pheromone signal peptide, normally expected to be excised during the translocation process, into a membrane-spanning segment. A similar shift of the signal peptide to an internal position is known to occur also in the generation of nerve growth factor isoforms, characterized by a longer amino terminus due to the maintenance in frame of an IVS (21).

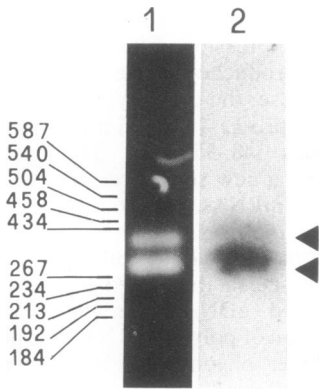

FIG. 3. Application of the RT–PCR procedure to poly(A)$^+$ RNA of type I cells. Lane 1, ethidium bromide staining of the 1.5% agarose gel loaded with the total amplified product; lane 2, autoradiograph of the same gel blotted and hybridized with the labeled insert of clone p5/6. At left, positions of size markers are indicated in base pairs; at right, the two amplified bands are indicated by arrowheads.

The interior location of this hydrophobic segment is illustrated in the hydrophobicity profile (22) shown in Fig. 4. The two shorter hydrophobic segments aligned in tandem at the amino terminus may serve in some way as signal peptides for the translocation of the larger molecule across membranes, although they show neither the expected length nor the other characteristics of signal peptides (or transmembrane segments). This translocation (or insertion into membranes) might be controlled by the signal sequence of prepro-E*r*-1, now located internally in the protein encoded by clone p5/6. An internal transmembrane segment and the lack of an amino-terminal signal peptide are indeed characteristic of transmembrane proteins oriented with the carboxyl terminus on the outside of the cell (23).

To obtain evidence that the clone p5/6 product is exposed in cell membranes, a polyclonal E*r*-1 antiserum was used in immunoblot analysis of detergent-solubilized membrane proteins of type I cells. One major band at M_r 14,000 was consistently evident; occasionally, a minor band at M_r 28,000 was seen (Fig. 5). The former corresponds to the calculated value for the product of clone p5/6 and the latter to that of the dimeric form of the M_r 14,000 subunit, which is assumed to remain associated by means of hydrophobic interactions

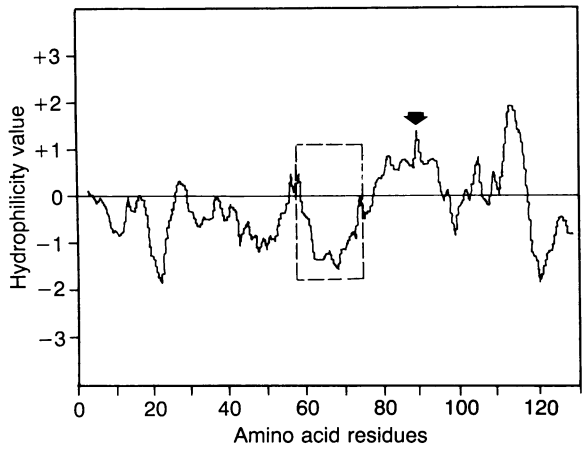

FIG. 4. Hydropathy profile of Er-1mem, predicted using a window size of six residues. The profile was obtained using the Apple DNA Inspector II software. Arrow indicates the amino terminus of secreted Er-1. The hydrophobic domain corresponding to the putative signal peptide of secreted Er-1 is boxed.

resistant to SDS solubilization. These same bands, plus two others corresponding to the sizes of Er-1 precursor and Er-1, were produced also by total cell extracts.

These results support the cell surface membrane location of the p5/6 clone product, which we have consequently designated Er-1mem. In addition, the observation (data not shown) of a band at M_r 14,000 in immunoblot analyses of membrane preparations of type II and type X cells, which produce pheromones (Er-2 and Er-10, respectively) that are similar in sequence to Er-1 (8, 24) and that are recognized by Er-1 antibodies (25, 26), suggests that soluble and membrane-bound pheromone forms may be produced by *E. raikovi* in different cell types.

What Is the Origin of Er-1 and Er-1mem Transcripts? The identity of Er-1 and Er-1mem cDNA sequences (except in the IVSs) strongly suggests that both these forms are controlled by the same gene through a mechanism of alternative elimination of the 5′[p5/6] and 3′[p45/2] IVSs. This mechanism may involve either a DNA rearrangement with the formation of two DNA molecules in the transcriptionally active macronucleus from a common precursor in the germinal nucleus (micronucleus) or, as it occurs in the generation of membrane-bound and secreted forms of neural cell adhesion molecule (27), immunoglobulins (28), and nerve growth factor (21) in mammalian cells, a posttranscriptional RNA splicing. In principle, the former alternative appeared to be favored. Indeed, the 5′[p5/6] and 3′[p45/2] IVSs do not show junctional consensus sequences of classical introns (29) and, in addition, they structurally match the "internal eliminated sequences" (IESs) originally described in the micronucleus of *Oxytricha nova* (30). These IESs are precisely removed from the developing macronuclear genome (31–33) and, like *E. raikovi* IVSs, contain short direct repeats at the junctions and internal inverted repeats. Experimental evidence, however, did not support the presence of two macronuclear DNA molecules, as required by a mechanism of DNA rearrangement; rather, it suggested that the complete information for both Er-1 and Er-1mem is contained in one DNA molecule.

Native macronuclear DNA of type I cells (represented by overamplified linear gene-sized molecules) was first probed in a Southern blot analysis with the insert of clone p5/6. Only one band of 1100 bp, coincident with that identified previously (11) as specific for the secreted Er-1, was revealed (Fig. 6, lane 1) and shown to be produced by molecules containing the 3′[p45/2] IVS. Autoradiographs of gels loaded with DNA that had been preincubated with *Hin*cII enzyme, which recognizes one specific site in the 3′[p45/2] IVS, revealed in fact one band of reduced size (850 bp). Remnants of the original band were visible only in overexposed gels and were presumed to represent low amounts of the same undigested molecular species (rather than a second molecular species lacking the *Hin*cII site).

The same DNA was then subjected to PCR amplification, initiated either with the two oligonucleotides used as primers in the RT–PCR procedure or by introducing as a 3′ primer a 21-mer oligonucleotide corresponding (as shown in Fig. 2*A*) to a sequence starting five nucleotides downstream of the 3′[p45/2] IVS. Only one product was generated (Fig. 7). This product was directly sequenced and shown to contain the sequence of clone p5/6 from the 5′ primer to nucleotide 578 (including the 5′[p5/6] IVS), the complete sequence of the 3′[p45/2] IVS, and the short 3′ region common to both clones p5/6 and p45/2.

Er-1mem Function. Why do *E. raikovi* cells produce Er-1mem in addition to secreted Er-1 and encode both these proteins by a single gene in the micronucleus and/or the macronucleus? As has been suggested for the epidermal growth factor and transforming growth factor α precursors

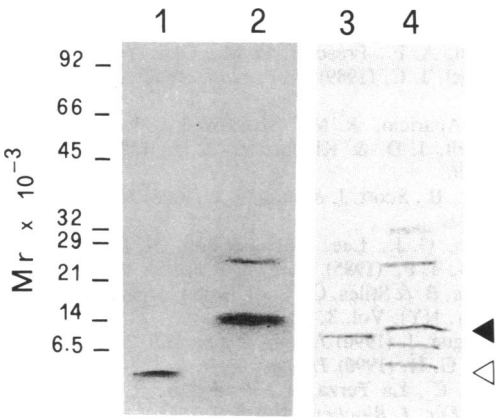

FIG. 5. Immunoblot analysis of membrane proteins of type I cells with anti-Er-1 rabbit polyclonal antibodies. Lanes: 1, purified Er-1 (8 μg); 2 and 3, solubilized membranes (50 and 30 μg, respectively); 4, total cell extract (50 μg). Lanes 1 and 2 were blotted on nitrocellulose membrane, and lanes 3 and 4 on Immobilon membrane. At right, solid and open arrowheads indicate a band of M_r 14,000 (i.e., of the size calculated for Er-1mem) and the band produced by Er-1. Positions of molecular weight markers are indicated at left.

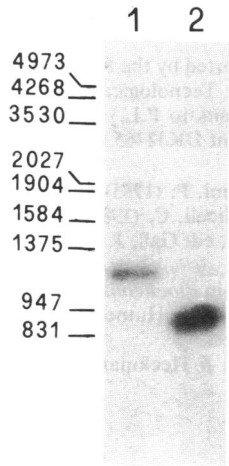

FIG. 6. Southern blot analysis of macronuclear DNA of type I cells. Undigested DNA (lane 1) and DNA digested with *Hin*cII (lane 2) were electrophoresed in a 1% agarose gel, blotted, and hybridized with the labeled insert of clone p45/2. Positions of markers are indicated in base pairs.

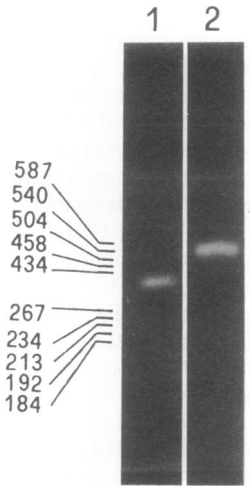

FIG. 7. Application of the PCR procedure to macronuclear DNA of type I cells. The oligonucleotides used correspond to sequences of clone p5/6 indicated in Fig. 2*A*. The 1.5% agarose gel was stained with ethidium bromide. Lane 1, amplified product obtained using the 21-mer and 29-mer oligonucleotides upstream and downstream, respectively, of the 5'[p5/6] IVS; lane 2, amplified product obtained using the 21-mer oligonucleotide upstream of the 5'[p5/6] IVS and the 21-mer oligonucleotide downstream of the 3'[p45/2] IVS. At left, positions of markers are indicated in base pairs.

when they are not processed (34–36), Er-1mem may act as a binding site for secreted Er-1. Such an activity would require that Er-1mem is inserted into the membrane with the carboxyl-terminal domain on the outside of the cells, a likely possibility (23) as judged by the lack of an amino-terminal signal peptide and a single, internal transmembrane segment.

That Er-1mem may bind secreted Er-1 is consistent with the property of the secreted, biologically active form of *E. raikovi* pheromones to occur as tightly bound homodimers (or higher aggregates) (10, 12) and the ability of these pheromones to act in an autocrine fashion (5). It is also in accord with analyses of mating-type inheritance in *Euplotes* species, which suggest that the complete genetic information for the pheromones and their receptors lies in a single locus (1, 2, 37). Should this model prove correct, the often-expressed hypothesis that polypeptide hormones and their receptors arose from a common molecule whose genetic and functional domains became separated during evolution would be substantiated by direct experimental evidence.

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