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Genetics

Reordering of nine exons is necessary to form a functional actin gene in *Oxytricha nova*

(macronuclear development/micronucleus/hypotrichs)

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ABSTRACT **During the development of a macronucleus from a micronucleus after cell mating in hypotrichs all the genes (≈20,000) are excised from micronuclear chromosomes as individual small DNA molecules. Telomeres are added to the ends of each gene-sized molecule and each is amplified, mostly by ≈1000-fold, to yield a transcriptionally active macronucleus. As a part of the study of the excision of genes from chromosomes, we have cloned six fragments of chromosomal DNA from *Oxytricha nova*, each containing a full copy of an actin gene, for comparison with the structure of the actin-encoding DNA molecule in the macronucleus. All six micronuclear actin clones had the same overall organization as judged by restriction mapping. Two micronuclear actin clones were sequenced. These differ from one another at a few nucleotide positions but both prescribe precisely the same actin polypeptide. Both micronuclear actin genes contain nine exons separated by eight intron-like sequences. The macronuclear gene contains these nine exons without intron-like segments. Assigning the order 1 through 9 to the nine micronuclear exons, the order in the macronucleus is 8–7–1–2–4–3–5–9–6. In the micronuclear actin gene, all nine exons possess terminal repeat sequences. These repeat sequences provide precise directions for reordering and joining of the nine exons to yield the exon order in the macronuclear gene. Polymerase chain reaction analysis of micronuclear DNA of the related species, *Oxytricha trifallax*, shows that the actin gene has an unorthodox arrangement in this species also.**

The genetic apparatus of hypotrichous ciliates consists of two morphologically and functionally different nuclei, the micronucleus and the macronucleus. The micronucleus contains high molecular weight DNA organized into chromosomes that divide mitotically and undergo meiosis during the sexual phase of the organism's life cycle (1). In contrast, the macronucleus contains only low molecular weight DNA molecules, ranging in size from ≈500 base pairs (bp) to 15 kilobase pairs (kbp) with a number average size of 2200 bp (2). There are ≈20,000 different molecules, and each appears to contain only one transcription unit or gene-encoding region. Each of the 20,000 molecules is present, on average, in ≈1000 copies (3). Transcription is very active in the macronucleus but is not detectable in the micronucleus. The micronucleus is not essential for vegetative growth in at least some species of hypotrichs, including *Oxytricha nova*.

The micronucleus functions as a germ-line nucleus. When two cells mate, the micronucleus divides meiotically, and haploid micronuclei are exchanged between the paired cells. An exchanged nucleus fuses with a stationary haploid micronucleus to form a new diploid zygotic micronucleus. Shortly thereafter the old macronuclei and the remaining haploid micronuclei are destroyed. After conjugation, the new diploid micronucleus divides mitotically without cell division. One of the two new micronuclei develops to a new macronucleus.

Our long-term objective is to find out how hypotrichs excise their genes from chromosomes during macronuclear development. We know that genes occur in micronuclear chromosomes singly or in small clusters separated by long spacers (4, 5). The formation of a macronucleus from a micronucleus is a complex process that includes polytenization of the chromosomes, destruction of the polytene chromosomes, degradation of all repetitive sequences and 90–95% of all unique sequences, elimination of intron-like sequences (ILSs), and addition of telomeric sequences to the gene-sized DNA molecules (6). A gene-sized molecule possesses a coding region, a short leader sequence, and a trailer sequence.

MATERIALS AND METHODS

DNA Cloning and Sequencing. A library of micronuclear DNA molecules was constructed in phage λ47.1 as described (7). A deletion series of subclones was generated for double-stranded DNA sequencing by the Erase-A-Base system (Promega). Dideoxynucleotide sequencing of double-stranded DNA was performed using the modified T7 DNA polymerase Sequenase (United States Biochemical) according to manufacturer's directions.

Southern Hybridization. Micronuclear DNA was digested with BAL-31 Fast Form (International Biotechnologies) for 20 min at 30°C, according to manufacturer's directions. Digested micronuclear DNA and cloned DNAs were electrophoresed in 1% agarose gels at 5 V/cm for 2.5 hr in 50 mM Tris borate, pH 8.0/2 mM EDTA. DNA was transferred to nitrocellulose overnight with 10× SSC as a transfer medium (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.4). Blots were prehybridized in 1 M NaCl/50 mM Tris-Cl, pH 8.0/1 mM EDTA/10× Denhardt's solution/0.1% SDS/sheared herring DNA (200 mg/ml) at 68°C for 4 hr (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin). Hybridization was carried out in a fresh prehybridization solution at 68°C for 16 hr. Post-hybridization washes were at a final stringency of 0.2× SSC/0.1% SDS at 68°C for 1 hr. Filters were air-dried and autoradiographed. The appropriate restriction fragment to serve as a probe for each hybridization was isolated by electrophoresis and labeled with ^{32}P by random-hexamer-primer extension to a specific activity of $>10^8$ dpm/μg.

Polymerase Chain Reaction (PCR) and Sequencing. A PCR was performed on 1 μg of native micronuclear DNA for 30

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Abbreviations: PCR, polymerase chain reaction; ILS, intron-like sequence.

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cycles with *Thermus aquaticus* (Taq) I polymerase (Perkin–Elmer/Cetus) essentially according to the manufacturer's instructions. For each successive cycle of replication, DNA was denatured at 95°C for 1 min, annealed to primers (100 μg/ml) at 42°C for 2 min, and extended at 72°C for 3 min. The PCR product was separated from the remaining micronuclear DNA and primers by electrophoresis on a 1% agarose gel. A second round of amplification was performed on the PCR product for 25 cycles at the same temperatures with primer 1 at 100 μg/ml and primer 2 at 1 μg/ml. This PCR product was purified on a NENsorb column (New England Nuclear) and sequenced essentially as above except that the primer (primer 3) was ^{32}P-5′-end-labeled and the DNA labeling step was omitted. A 12% polyacrylamide sequencing gel was used.

RESULTS

Arrangement of Sequences in Cloned Micronuclear and Macronuclear Actin Genes. The primary objective was to compare the macronuclear actin gene with the micronuclear copy from which it was derived. For this purpose the actin gene is defined as the 1532-bp molecule plus two 36-base telomeres that encodes the actin polypeptide in the macronucleus of *O. nova*. The gene consists of a 1128-bp actin-encoding region, a 191-bp leader sequence, a 213-bp trailer sequence, and a telomere sequence of 36 bases on each end. The complete sequence and other properties of the cloned macronuclear actin gene have been described (ref. 8 and EMBL/GenBank data base, accession no. M22480).

Six independently derived micronuclear DNA clones containing the actin gene were selected by hybridization from a micronuclear DNA library using the cloned macronuclear actin gene as the selector probe. The restriction nuclease maps and Southern blot hybridization were the same for all six clones, showing that they all had the same overall organization. Two clones, mic-act-1 and mic-act-2, were sequenced.‡ The organization of mic-act-1 and mic-act-2 is shown in Fig. 1. Both contain the entire 1532-bp actin gene sequence (leader, coding region, and trailer) plus adjacent sequences. No telomere sequences are present at either end of the actin gene in mic-act-1 or mic-act-2. Telomeres are added to the actin gene during macronuclear development after excision of the gene from the chromosome. In both micronuclear clones the left flanking region consists of a spacer of 15 bp that separates the actin gene from the next gene in the chromosome. This adjacent gene is called the 3.3-kbp gene on the basis of its size as it occurs in the macronucleus. Its coding function is unknown. Mic-act-1 and mic-act-2 differ at four positions in the 15-bp spacer between the two genes. The flanking sequence at the right end of the cloned actin genes is ≈1000 bp long. Mic-act-1 and mic-act-2 differ in 1.3% of their base pairs but both encode identical actin proteins.

The overall sequence organization within the actin gene itself in mic-act-1 and mic-act-2 is the same. There are nine exons separated by eight ILSs. These ILSs have been named internal eliminated sequences (IESs) (7). They are eliminated, apparently by cutting and splicing of DNA, during macronuclear development rather than cutting and splicing of the corresponding RNA molecules after transcription of the gene. The lengths of the nine exons and the ILSs that separate them are given in Fig. 2*a*.

The linear arrangement of the nine exons both in the cloned and in native macronuclear actin gene is shown in Fig. 2*b*. In the macronuclear actin gene the exons are in a different order than in the cloned mic-act genes. In addition, exon 7 is reversed in polarity. Exon 8 forms the first part (left end) of the macronuclear actin gene (transcription is from left to right). It makes up most of the leader sequence of the macronuclear gene and contains the site(s) at which transcription is initiated (8). It has the usual telomere sequence attached to its left end, acquired during macronuclear development. Exon 8 in the macronuclear gene is followed by exon 7, however, with reversed polarity. The adenine and thymine in the translation initiation codon ATG for the actin polypeptide are the last two bases of exon 8 and the guanine is the first base in the left end of exon 7 in the macronuclear gene. The remaining seven exons in the macronuclear gene follow exon 7 in the order, 1, 2, 4, 3, 5, 9, and 6, all in the same polarity as in the two mic-act genes. This order is achieved by interposition of exon 4 between exons 2 and 3 and interposition of exon 9 between exons 5 and 6. The translation stop codon TGA is located a short distance to the left of the right end of exon 3. Exons 5, 9, and 6 follow exon 3 and form the bulk of the trailer sequence of the macronuclear actin gene. A telomere sequence is joined to the right end of exon 6 during macronuclear development.

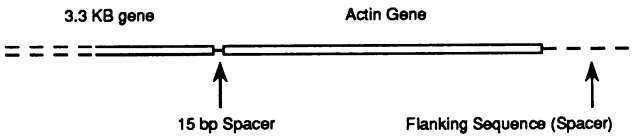

FIG. 1. Overall organization of the mic-act-1 and mic-act-2 clones. The actin gene and the 3.3-kbp gene are separated by a 15-bp spacer. The flanking region to the right of the actin gene continues for at least 1000 bp without evidence of another macronuclear gene.

Repeat Sequences in the Cloned Micronuclear Actin Gene. All nine exons of the micronuclear actin gene possess either direct or inverted sequence repeats at their ends, as shown schematically in Fig. 3. The sequences of the various direct and inverted repeats are given in Table 1. The first 13 bp of exon 1 make a sequence that is repeated in inverted orientation at the left end of exon 7 in mic-act-1 and mic-act-2. Homologous recombination between these inverted repeats joins the original left end of exon 7 with the left end of exon 1 and reverses the original polarity of exon 7. This is the exact arrangement observed in the cloned macronuclear actin gene. Similarly, the original right end of exon 7 consists of a 12-bp sequence that is an inverted repeat of the 12-bp sequence on the right end of exon 8. Homologous recombination between these invested 12-bp repeats, with elimination of one copy, joins the right end of exon 8 to the new left end of exon 7. ILS 7 between exons 7 and 8 is eliminated, and the left end of exon 8 serves for addition of a telomere sequence. The two homologous recombinations just described would yield the order 8–7–1 for the three exons which is the order found in the cloned macronuclear actin gene. Reordering of exons 7 and 8 is also accompanied by elimination of ILS 6, leaving the right end of exon 6 available for telomere addition.

Exons 1 and 2 in the mic-act genes are separated by ILS 1 (28 bp). The sequence AATC is directly repeated at the two junctions of ILS 1. Homologous recombination between these repeats would eliminate the ILS, eliminate one copy of the repeat, and join the right end of exon 1 with the left end of exon 2 to yield the same sequence found in the macronuclear clone. Exons 3, 4, 5, 6, and 9 could be spliced in the proper macronuclear order if homologous recombination were to occur between the two repeats in each pair of repeats, as shown in Fig. 3.

The direct and inverted repeats in the mic-act genes, therefore, indicate how through homologous recombination, the nine exons in the micronuclear actin gene can be reordered and joined with the accompanying elimination of the eight ILSs to yield the macronuclear gene sequence. In this

‡The micronuclear actin sequences reported in this paper have been deposited in the GenBank data base (accession nos. M25530 and M25531.

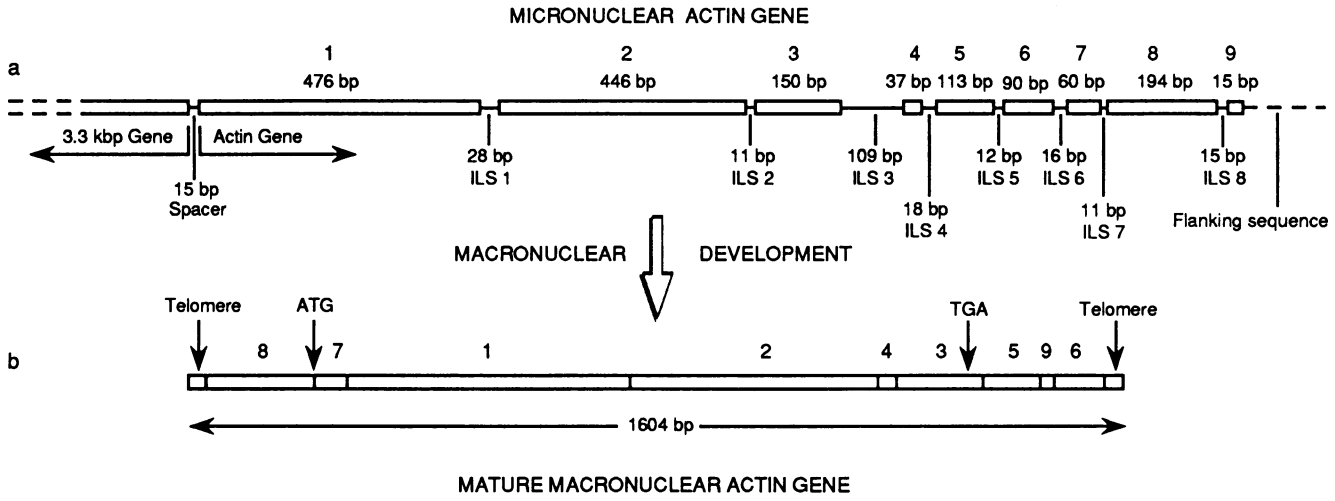

FIG. 2. Scale drawing showing the order and relative sizes of actin exons and ILSs in the micronuclear actin gene (*a*) and the macronuclear actin gene (*b*). Each rectangle depicts one exon. The size of each exon (in bp) was determined by counting from the first unique base pair in an exon through the direct repeat at the right end of the exon. Exon 7 was counted from right to left. ATG of the start codon is at the junction of exons 7 and 8 in the macronuclear gene.

scheme, the origin of every one of the 1532 bp of the macronuclear gene is precisely accounted for.

We have determined (8) the sequence of a macronuclear actin gene. That sequence matches perfectly the actin sequence generated by reordering of the nine exons in mic-act-2. Therefore, we conclude that mic-act-2 is processed to yield a macronuclear actin gene. We do not yet know whether mic-act-1 is processed to yield a macronuclear actin gene.

Order of the Nine Exons in the Actin Gene in Native Micronuclear DNA. Although the same unorthodox arrangement of exons was found by restriction nuclease mapping in all six micronuclear actin clones, the unorthodox arrangement could be the result of a complicated and consistently repeated artifact of cloning. To test the possibility of a cloning artifact, a portion of the actin gene in native micronuclear DNA was amplified by the PCR (see Fig. 4). The sequence of this PCR product was determined and compared to the sequence of the cloned DNA. The sequence of the PCR product generated in this way matched the sequence of clones mic-act-1 and mic-act-2. Thus, the order ILS 5, exon 6, inverted exon 7, ILS 7, and left end of exon 8 was confirmed for native uncloned micronuclear DNA. We conclude that the unorthodox arrangement of exons in the cloned mic-act genes is not an artifact of cloning and that the unorthodox arrangement is present in native uncloned micronuclear DNA.

Because of the orientation of the primers used in this PCR, any orthodox (macronuclear) arrangement present would not be detected. However, to test the possibility that there may be actin sequence arrangements other than the arrangement found in the micronuclear DNA clones and PCR products, the following was done: Nine micrograms of micronuclear DNA was treated with BAL-31, a procedure that reliably removes contaminating macronuclear actin sequences.

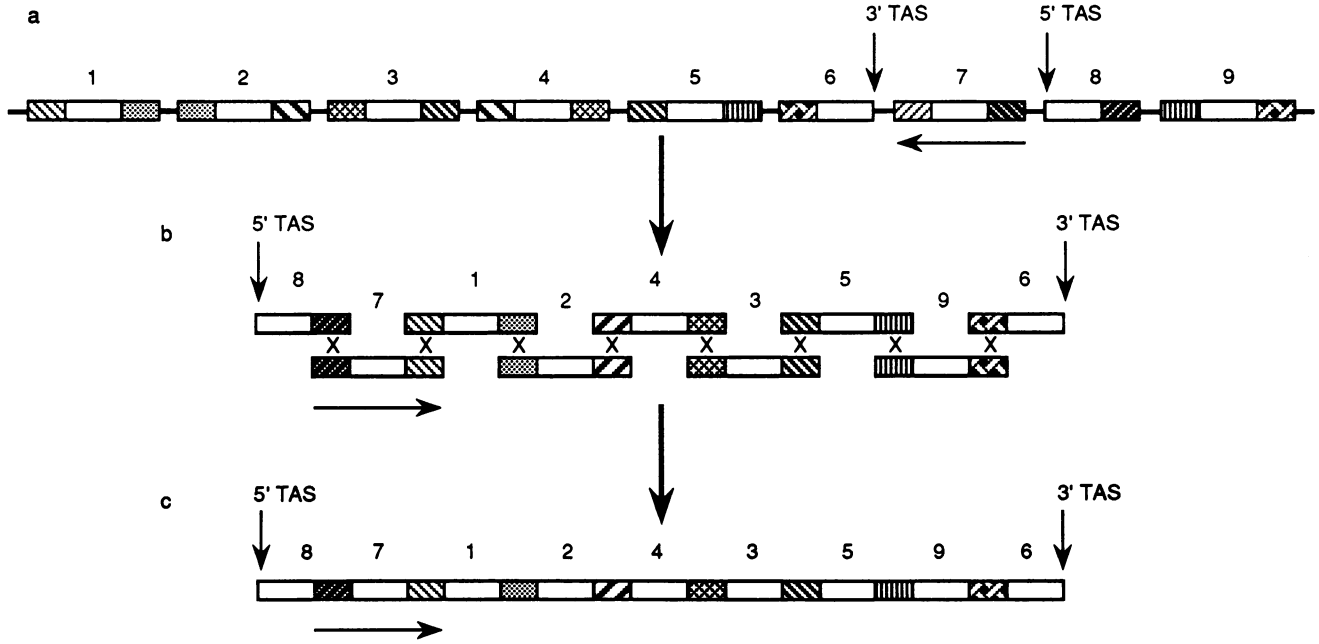

FIG. 3. (*a*) Arrangement of the direct and inverted repeats located at the ends of each exon. The blocks filled with various patterns represent pairs of repeats. (*b*) All exons can be arranged in the order of the macronuclear gene by aligning a given repeat with the other member of its pair. (*c*) Homologous crossing-over between the two members of each pair of repeats yields a complete macronuclear actin gene (except for telomeres) with ILSs removed. The polarity of exon 7 is shown by a horizontal arrow. TAS, telomere addition site.

Table 1. Direct and inverted repeats bordering each exon

Left-end repeat	Exon	Right-end repeat
GGAGTCGTCAAG	1	AATC
AATC	2	CTCCCAAGTCCAT
GCCAGCCCC	3	CAAAACTCTA
CTCCCAAGTCCAT	4	GCCAGCCCC
CAAAACTCTA	5	CTTTGGGTTGA
AGGTTGAATGA	6	3′ TAS
CTTGACGACTCC	7	ATGTGTAGTAAG
5′ TAS	8	CTTACTACACAT
CTTTGGGTTGA	9	AGGTTGAATGA

Direct and inverted repeats located at the ends of the nine exons in mic-act-1 and mic-act-2. The sequence at the left end of exon 7 is an inverted repeat of the sequence at the left end of exon 1, and the sequence at the right end of exon 7 is an inverted repeat of the sequence at the right end of exon 8. Homologous crossing-over between each pair of repeats reorders the exons into the order found in the macronuclear gene—i.e., 8–7–1–2–4–3–5–9–6.

Three micrograms of the BAL-31-treated DNA was digested with *Kpn* I, and the remainder was digested with *Kpn* I plus *Dra* I. The locations of the *Kpn* I and *Dra* I sites are shown in Fig. 4*a*. One lane of *Kpn* I-digested DNA and two lanes of *Kpn* I–*Dra* I-digested DNA were separated by electrophoresis on a 1% agarose gel and transferred to nitrocellulose. These blots of uncloned micronuclear DNA were tested with three probes prepared from digests of the cloned macronuclear actin gene (Fig. 4*b*). Probe 1 is specific for the 5′ end of the macronuclear actin gene, probe 2 is specific for a region in the middle of the actin gene and to the left of the single *Kpn* I site in the macronuclear actin gene, and probe 3 is specific for the 3′ end of the actin gene. The probes were checked for specificity by Southern hybridization to blots of DNA of cloned mic-act-2 digested with *Kpn* I plus *Dra* I. All probes hybridized only to the bands predicted from the map in Fig. 4*a*. Probe 2 hybridized to a 4.5-kbp *Kpn* I fragment and probes 1 and 3 hybridized to a 960-bp *Kpn* I–*Dra* I fragment.

The blot of *Kpn* I-digested uncloned micronuclear DNA was hybridized with probe 2. The uncloned micronuclear DNA digested with *Kpn* I plus *Dra* I was hybridized with probe 1 and probe 3 in separate experiments. The resulting autoradiogram of the Southern hybridization showed that only the bands predicted from endonuclease mapping and sequence determination of the micronuclear DNA clones were detected (see Fig. 4*a*). Probe 1 and probe 3, which represent the 5′ and 3′ end of the macronuclear actin gene, respectively, hybridized exclusively to the 960-bp *Kpn* I–*Dra* I fragment predicted from the sequence analysis of mic-act-1 and mic-act-2. Probe 2 hybridized only to the 4.5-kbp *Kpn* I fragment predicted from restriction endonuclease analysis of the mic-act genes. Since probe 1 hybridized only to the same band to which probe 3 hybridized and did not hybridize to the band to which probe 2 hybridized, we conclude that no orthodox arrangement of actin exons is present in micronuclear DNA. If there were a micronuclear actin gene with the orthodox arrangement of exons as seen in the macronuclear actin gene, then both probe 1 and probe 2 would have hybridized to the same *Kpn* I–*Kpn* I restriction fragment. Furthermore, since no unpredicted bands were found, there are no sequence arrangements other than the arrangement found in the micronuclear DNA clones and the PCR product obtained with micronuclear DNA.

Micronuclear Actin Gene in *Oxytricha trifallax*. It is conceivable that during the 8-year interval since our strain of *O. nova* last mated that the micronuclear actin genes have rearranged into the unorthodox order we have detected and do not represent the form in cells with a normal micronucleus capable of successful mating. Therefore, we examined micronuclear actin DNA in *O. trifallax*, for which we have mating strains (a gift from Robert Hammersmith, Ball State University). Two clones of *O. trifallax* were grown and mated, with >90% survival of exconjugants. Micronuclei were isolated from one of the parental strains and the DNA was used in a PCR. Primers for the reaction were synthesized using the sequence of the macronuclear actin gene published for a similar species, *O. fallax* (9). Primers were chosen to test for the presence of an unorthodox arrangement of the actin gene in micronuclear DNA. A PCR with these primers using total macronuclear DNA of *O. trifallax* or a cloned macronuclear actin gene from *O. fallax* predictably yielded no

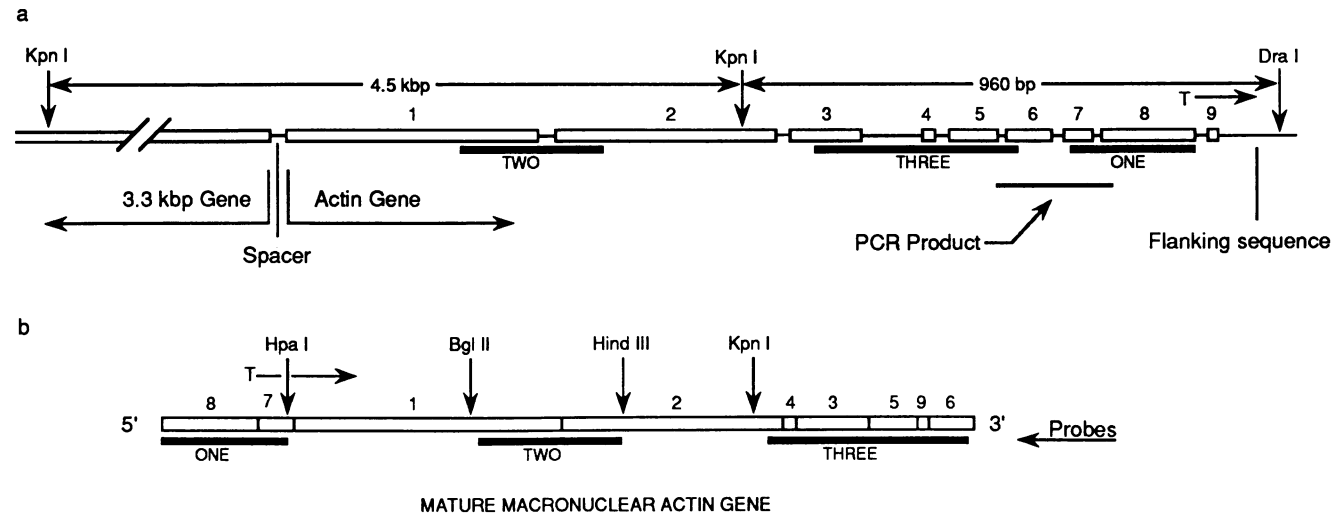

FIG. 4. Relationship of the three hybridization probes (solid thick bars) derived from cloned macronuclear actin DNA to the two restriction fragments of actin-specific native (uncloned) micronuclear DNA produced by *Kpn* I plus *Dra* I digestion. Probes 1 and 3 are derived from the 5′ and 3′ ends of the macronuclear actin gene, respectively. Probe 2 is derived from the *Bgl* II–*Hin*dIII fragment located to the left of the *Kpn* I site in the macronuclear actin gene. If the exon arrangement of the macronuclear actin gene was the same in native micronuclear DNA, then probe 1 would hybridize to the same *Kpn* I–*Kpn* I restriction fragment in the micronuclear actin gene as probe 2 (see *b*); however, it does not. Instead, probe 1 hybridizes to the same restriction fragment as probe 3 and only to that fragment. Therefore, the arrangement of exons in native micronuclear actin DNA is different from the arrangement in the cloned macronuclear actin gene. The line labeled PCR product indicates the region from ILS 5 to the left end of exon 8 that was amplified from native micronuclear DNA by a PCR and sequenced. The capitol T over exon 8 in *a* indicates the transcription start site, and the arrow shows the direction of transcription that would occur if micronuclear DNA were to be transcribed.

product. A PCR with these primers using total *O. trifallax* micronuclear DNA or total *O. trifallax* micronuclear DNA digested with BAL-31 to remove macronuclear DNA contamination yielded an abundant product of ≈630 bp. A PCR could only yield such a product if the exons containing the primer sequences were in an unorthodox arrangement in micronuclear DNA of *O. trifallax*. Since this shows an unorthodox order of exons in *O. trifallax*, we conclude that the unorthodox order in *O. nova* is not an artifact that arose during the many years since its last mating.

DISCUSSION

The actin gene or genes in the micronucleus of *O. nova* consist of nine exons, arranged in an unorthodox fashion. The exons are reordered to form a functional actin gene during macronuclear development. These statements are based of the following observations: (*i*) Six micronuclear DNA clones containing complete micronuclear actin genes were isolated. All six were shown by restriction mapping and Southern hybridization to have the same unorthodox arrangement. Two micronuclear actin genes, mic-act-1 and mic-act-2, were defined by minor differences in sequences. (*ii*) The unorthodox arrangement of the nine exons is not a cloning artifact because the same arrangement was shown to be present in uncloned micronuclear DNA using the PCR method. This was confirmed with three segments of the cloned macronuclear actin gene as hybridization probes to test the order of exons in uncloned micronuclear DNA. (*iii*) No orthodox versions of the actin gene could be found in uncloned micronuclear DNA using the three segments of the cloned macronuclear actin gene as search probes. (*iv*) Macronuclear actin genes contain only the orthodox arrangement of exons. (*v*) An unorthodox arrangement of the actin gene is not unique to *O. nova* but also occurs in *O. trifallax*.

The presence of direct and inverted repeat sequences at the ends of the nine exons in the micronuclear actin gene indicates a precise scheme for generating the macronuclear actin gene for the micronuclear gene. Recombination between the two members of each pair of repeats would create the orthodox order and eliminate all ILSs, as shown schematically in Fig. 3. For exons 1 and 2, a direct repeat of 4 bp (AATC) occurs at each junction with the ILS between them and after removal of the ILS; one of these repeats remains in the junction between the two exons. The remaining seven ILSs are bordered by longer repeats, ranging from 9 to 13 bp, and these ILSs separate exons that become reordered. In these seven cases the removal of the ILS is accompanied by a transposition of an exon to join (presumably by homologous recombination) an exon other than the one originally adjacent. Thus, short direct repeats are present in the one case where two adjacent exons are joined (exons 1 and 2), and the intervening ILS is removed. Where exons are transposed, longer repeat sequences lie at the ILS junctions.

Construction of the macronuclear actin gene could conceivably occur by transcription of the micronuclear actin gene in the developing macronucleus. The transcript could be processed to remove ILSs and reorder exons, followed by conversion into DNA by reverse transcriptase (10, 11). This would require transcription of portions of both strands of the micronuclear actin DNA. In generating the macronuclear gene the coding strand is derived from one strand of exons 1–6, 8, and 9 and from the opposite strand for exon 7. Therefore, at least portions of both strands of the micronuclear gene must be transcribed and ILSs must be removed. Eight RNA segments transcribed from one strand and one exon in a transcript of the opposite strand must be spliced together to produce an RNA suitable for reverse transcription into a DNA molecule to which telomeres are added to form the macronuclear version of the actin gene. We favor a DNA-processing model since it seems molecularly less demanding than the RNA model.

Micronuclear and macronuclear versions of three genes in *O. nova* have been analyzed by Ribas-Aparicio *et al.* (10). They have ILSs with short direct repeats at their junctions in the micronuclear versions of the genes. The short direct repeats presumably signal correct excision and splicing, and ILS 1 in the actin genes described in this paper is similarly organized. However, Ribas-Aparicio *et al.* (10) found no sign of reordering of exons in any of the three micronuclear genes to make the macronuclear versions. Two extensions of the present work are to test for unorthodoxy among other genes in *O. nova* and to test whether unorthodoxy occurs in hypotrichs generally.

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