Tetrahymena conjugation-induced genes: structure and organization in macro- and micronuclei

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Received 8 October 1985; Revised 12 December 1985; Accepted 20 December 1985

ABSTRACT

The physical organization of eight *Tetrahymena* genes active during conjugation (meiosis) was examined in the somatic (macro-) and germinal (micro-) nuclei of this organism. Three of these genes make transcripts that are only detected during meiotic prophase. Southern blot analyses indicated that all genes examined were present in both nuclei. Except for one gene family (pC6), all appeared to be non-repetitive and there were no detectable sequence rearrangements in or near the genes. The exceptional gene was repeated approximately five to seven times and DNA rearrangement occurred in or near each of these copies. A comparison of cDNA and macro- and micronuclear DNA restriction maps indicated that one of the genes (cnj B) contains introns. This is the first report of evidence for introns in a non-ribosomal gene in *Tetrahymena*. A site specific modification probably due to adenine methylation was seen in the macronuclear copy of another gene (cnj C).

INTRODUCTION

Tetrahymena thermophila, a ciliated protozoan, contains within a single cell two functionally distinct nuclei (1). During vegetative growth, the polyploid macronucleus (somatic nucleus) is transcriptionally active; its genes determine the phenotype of the cell. The diploid micronucleus (germinal nucleus) is transcriptionally silent. Under proper conditions, cells can be induced to undergo conjugation very synchronously; the micronuclei proceed through meiosis, cross-fertilization and the formation of new macro- and micronuclei while the old macronucleus is degraded (2). During the formation of the new macronucleus approximately 10% to 20% of the micronuclear genome is eliminated (3). This elimination involves extensive breakage and rejoining of DNA at defined sites (4). So far the physical arrangement of very few structural genes has been examined in the two nuclei (5, 6).

Genes active during conjugation have been isolated (7). Three of these genes are conjugation-specific. They make transcripts that are detectable

only during meiotic prophase, peaking in abundance just before the maximum elongation of the micronucleus (pachytene). This is very close to the only time when micronuclear transcription can be detected (8, 9). Since these genes are active only at this time, we looked to see if these genes might be among the sequences or associated with sequences eliminated from the macronucleus. Other clones reported in this study contained genes whose transcripts were present during conjugation as well as other times of the life cycle.

In this report we compare the physical arrangement of these conjugation associated genes in the macro- and micronuclei. Southern hybridizations of the genomic DNAs with cDNAs corresponding to the various genes indicate that all of the conjugation-specific genes are non-repetitive, are present in both nuclei, and that no DNA rearrangements occur within or nearby the genes. One of the conjugation-induced genes was both repetitive and located near DNA rearrangements. Evidence is presented for introns in one of the conjugation-specific genes as well as for a site-specific modification in the macronuclear copy of another.

MATERIALS AND METHODS

DNA

All micronuclear and macronuclear genomic DNAs were obtained from Dr. M.A. Gorovsky, University of Rochester. These DNAs were isolated from the diploid strain CU401, and the nullisomic strains CU358 (nulli 3, 4 and 5), CU359 (nulli 2, 3 and 5) and CU383 (nulli 4) (10). The cDNAs used have been described (7).

Gel Electrophoresis and Blotting

Restriction enzymes were purchased from Bethesda Research Laboratories (BRL) and used in approximately three-fold excess to ensure complete digestion. DNA was separated on 0.8% to 1.5% agarose gels (horizontal) in TBE buffer (89 mM Tris, 89 mM Boric acid, 2.5 mM Na₂EDTA, pH 8.3) at 3 to 4 V/cm for 3 to 4 hr. Both the running buffer and the gel contained 0.5 µg of ethidium bromide per ml. Size markers were obtained from BRL (lambda DNA digested with HindIII and φX174 RF DNA digested with Hae III). DNA was transferred from the gel to Gene Screen filters (New England Nuclear) by the method of Southern (11). Restriction fragments were isolated by electrophoresis onto and recovery from NA-45 DEAE membranes (Schleicher and Schuell) as described by the manufacturer.

DNA Labelling, Hybridization and Autoradiography

DNA was labelled by nick-translation (12) using a kit and ^{32}P -labelled dCTP (800 Ci/mmol) from Amersham Corporation. The filters were prehybridized for 4 hr to 16 hr at 42°C in a buffer containing 50% formamide, 5 X SSC, Denhardt's Solution (0.02% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 0.2% sodium dodecyl sulfate, and 10% dextran sulfate (Sigma). Labelled probes (ca. 10^6 cpm per filter) were denatured 5 min at 90°C to 100°C, mixed in one ml of prehybridization buffer and then added directly to the prehybridization mixture. Hybridization was carried out at 42°C for 12 to 20 hr. The filters were washed and exposed to X-ray film as previously described (13).

Reconstruction Lane

To determine whether the genes corresponding to the cDNAs pC1 and pC7 are repetitive or non-repetitive, a reconstruction lane was run next to lanes containing genomic DNA. The genomic lanes contained 4 µg of restriction enzyme-digested micro- or macronuclear DNA. The reconstruction lane contained a total of 4 µg of yeast DNA plus pC1 or pC7 DNA. Appropriate quantities of the cDNA plasmids to add were calculated by using the value of one genome equals 0.23 pg of micronuclear DNA (Gorovsky, 1980); thus 4 µg of micronuclear DNA would be equivalent to 1.7×10^7 genomes. The masses of 1.7×10^7 molecules of pC1-2 (6510 bp) and pC7-1 (5580 bp) were then calculated (121 pg and 104 pg respectively), added to 4 µg of yeast genomic DNA, and cut with the appropriate restriction enzymes. The DNAs were then run on gels, transferred to filters and hybridized as described elsewhere.

RESULTS

Sequences corresponding to eight *Tetrahymena* genes active during early conjugation have been identified in a cDNA library made from mRNA isolated during meiotic prophase (7). The names assigned to these genes are listed in Table 1 (see 7 for their transcription patterns). The arrangements of these genes in both the somatic (macro-) and germinal (micro-) nuclei were investigated by hybridizing labelled cDNA probes corresponding to these genes to restriction enzyme-digested micronuclear (m) and macronuclear (M) DNA (figure 1). Results using five of the eight classes of cDNA probes against both genomic DNAs digested with HindIII and Hha I are shown. Except for pC6, each probe (including the three not shown) hybridized to the same-sized restriction fragments in both nuclei, indicating that no major sequence elimination or rearrangement had occurred within or near these genes.

TABLE I - GENE NAMES AND LOCATIONS

Characteristic	Locus	cDNA	Micronuclear Chromosome
Conjugation-specific	cnj A	pC1	4
	cnj B	pC2	3 or 5
	cnj C	pC3	3 or 5
Conjugation-induced	cup A	pC3	See text
	cup B	pC4	4
	cup C	pC8	2
	multiple	pC6	4
Non-growth specific	ngo A	pC5	2

Figure 1. Comparison of Conjugation Genes in Macro- and Micronuclear DNA: Approximately 3 μ g each of macronuclear and micronuclear DNA were digested with HindIII and Hha I, separated on an agarose gel and blotted for hybridization. This figure shows the results obtained when various labelled cDNA probes were hybridized against macronuclear (M) and micronuclear (m) DNA. The standards (HindIII digested lambda) shown on the left correspond to the hybridizations with pC1, pC3 and pC4 while those on the right refer to those with pC6 and pC8.

Figure 2. Restriction Map Summarizing the Structure of *cnjB*: This map is based upon a number of Southern blots of macro- and micronuclear DNA and cDNA. The restriction maps of the two nuclear DNAs were the same. The cDNA plasmid pC2-1 was used. The Pst I sites at the ends of the cDNA molecule were constructed during their cloning (7). A: AccI, E: EcoRI, H: HindIII, h: Hha I, Hp: Hpa I, p: Pst I, X: Xho I (restriction sites were not found for BamHI, Bgl I, Kpn I, Pst I and Sal I).

Additional Southern hybridizations with the eight cDNAs and macro- and micronuclear DNA cut with additional restriction enzymes confirmed these results. More detailed analyses of the genomic DNA hybridizing to five of the cDNAs (pC1, pC2, pC5, pC7, and pC8) were done and genomic restriction maps for each (identical for the two nuclei) have been constructed (see, for example, figures 2 and 5). Except for pC6, the number and sizes of the restriction fragments corresponding to each probe is best accounted for by each probe hybridizing to a gene in a single location within the macro- and micronuclear genome. Single-copy reconstruction lanes were included in experiments with pC1 and pC7 to determine whether the autoradiographic signals seen with these probes were of the level expected of single copy genes. In both cases the signals seen from the hybridizations in the reconstruction lanes were approximately the same as the signals from the genomic lanes (data not shown; see Materials and Methods for experimental details).

The results from many Southern experiments using the eight different cDNA probes and various macro- and micronuclear genomic DNA preparations consistently showed that the intensity of the autoradiographic signals were slightly stronger in hybridizations against macronuclear DNA than in hybridizations against equivalent quantities of micronuclear DNA (the difference seen with pC3 and pC8 in figure 1 were not as exaggerated in subsequent hybridizations). These differences may be due to the higher gene

to total DNA ratio of the macronucleus that results from the elimination or under-replication of 10% to 20% of the micronuclear DNA sequences (3).

The recombinant plasmid pC6-1 (755 bp cDNA insert) hybridized to a family of genomic DNA restriction fragments (figure 1) but to only one RNA species (1470 bases: 7). When genomic DNA was digested with EcoRI, five to seven restriction fragments from either the micronuclear or macronuclear DNA hybridized to pC6; all of the fragment sizes in one nucleus were different from those in the other (data not shown). The smallest of these EcoRI fragments was 3100 bp. When macronuclear or micronuclear DNA was double-digested with HindIII and Hha I (figure 1), the two nuclei again had different sized restriction fragments hybridizing to pC6, but there was also a set of small fragments that were the same size in both nuclei (590, 540, 520, 355 and 250 bp). These results suggest that pC6 hybridizes to approximately five to seven related genes or pseudogenes and the sequences adjacent to these genes are changed during nuclear differentiation. These differences are unlikely to be due to differences in methylation (see discussion). The set of small HindIII/Hha I restriction fragments that are of equal size in the two nuclei likely represent sequences that are within or right next to the genes and remain unchanged during nuclear differentiation.

Nullisomic strains missing both members of various micronuclear chromosome pairs (10) were used to map the various genes to the five micronuclear chromosomes. Recombinant plasmids corresponding to the eight genes previously discussed (see Table 1) were labelled with ^{32}P and used to probe micronuclear DNA from *Tetrahymena* strains CU358 (missing chromosomes 3, 4 and 5), CU359 (missing chromosomes 2, 3 and 5) and CU383 (missing chromosome 4). If, for example, a gene was on chromosome 4, the plasmid corresponding to this gene would hybridize to micronuclear DNA from CU359 but not from CU358 or CU383. We could not differentiate between chromosomes 3 and 5 by using these strains. Table 1 lists the micronuclear chromosomal locations of the various genes. The only ambiguous result was obtained with cup A; the cDNA corresponding to this gene (pC3-2) hybridized to both CU359 and CU383 micronuclear DNA, but not to micronuclear DNA from CU358 indicating it is on both chromosome 4, and 3 or 5. Either there are two or more copies of this gene (Southern blotting indicates a single copy; see figure 1) or one or more of the nullisomic strains are not what they were originally thought to be (for example, CU359 may have retained a fragment of chromosome 3 containing cup A). The main point that can be made from this data is that there is no obvious

Figure 3. Evidence for the Correlation Between the Genomic and cDNA Restriction Maps of *cnj B*: A. DNA from the lambda-*Tetrahymena* genomic DNA clone 1C2-1 was digested with EcoRI (E) and HindIII (H) and separated in lanes 1 and 2 respectively. Similarly, DNA from the cDNA clone pC2-1 was digested with HindIII and EcoRI and separated in lanes 3 and 4 respectively. A filter containing this DNA was hybridized with the ^{32}P -labelled 2.4 kb HindIII fragment of 1C2-1 and the resulting autoradiograph is shown on the right. HindIII digested lambda DNA was used for size markers. B. This diagram shows the sizes (in kilobases) and the orientation of the HindIII (H) and EcoRI (E) restriction fragments in 1C2-1 and pC2-1. The broken double lines on the ends of pC2 represent the vector pBR322. Since pC2-1 is circular, the H and E sites in parenthesis at the right end of pC2 represent the same sites as the H and E sites at the left end.

clustering of these conjugation-specific or induced genes.

Clear differences were seen when the restriction maps of the cDNA contained in pC2-1 (3.6 kb) and the genomic DNA hybridizing to pC2-1 were compared (figure 2; macro- and micronuclear genomic DNA had the same restriction maps). A *Tetrahymena* genomic DNA library constructed by Craig Finley (Yale University) using total cellular DNA was screened with pC2-1, and several recombinant lambda clones containing cnj B gene sequences were isolated (1C2-1, 1C2-2 etc.). The restriction maps of these cloned genomic DNAs were identical to the restriction maps obtained using genomic DNA that was isolated directly from either the macro- or micronucleus, restriction enzyme-digested and probed with pC2-1. The construction of the restriction maps was facilitated by three restriction enzymes (Acc I, Hpa I and Xho I) that each cleaved both the cDNA and corresponding genomic DNA once (each cleaved a different genomic HindIII or EcoRI fragment). The correlation shown between the genomic DNA and cDNA maps in figure 2 was further confirmed by isolating from 1C2-1 the three genomic HindIII fragments that were hybridized by pC2-1, labelling these fragments, and demonstrating that they hybridized to the appropriate pC2-1 and 1C2-1 restriction fragments. Figure 3 shows the results of one such experiment. In this case the 2.4 kb HindIII fragment from 1C2-1 was isolated, labelled and hybridized against EcoRI or HindIII digested 1C2-1 or pC2-1. As can be seen in the autoradiograph in figure 3A, this fragment hybridized to the restriction fragments predicted from the restriction maps drawn in figures 2 and 3B. Similar experiments using the 1.7 kb and 2.9 kb genomic DNA HindIII fragments as probes also gave the expected results (for example, only the 1.7 kb 1C2-1 HindIII fragment hybridized to the 1.2 kb pC2-1 HindIII fragment; data not shown). The differences between the genomic DNA and cDNA restriction maps suggest that at least six introns are present within the cnj B gene (3.3 kb of cDNA corresponded to 7.0 kb of genomic DNA; a 235 bp HindIII/Acc I restriction fragment is the only one found to be the same size in both maps.) Since the cDNA of pC2-1 represents only about one-half of the cnj B transcript (7450 bases), the cnj B gene is expected to be very large (over 10 kb).

When cDNA pC7 (cnj C) was used as a probe against micronuclear and macronuclear DNA that had been digested with EcoRI alone or in combination with other restriction endonucleases, a 60 bp size difference was always seen between the two nuclei in one of the fragments (figure 4A). This difference was not due to an extra 60 bp being inserted into the macronuclear DNA since the difference was not seen when other restriction enzymes

Figure 4. Evidence for Macronuclear Modification of *cnj C* Gene: Approximately 3 μ g each of macronuclear (mac) and micronuclear (mic) DNA were digested with HindIII and EcoRI (A) or Taq I (B), separated on an agarose gel and blotted for hybridization. This figure shows the results of hybridizing the filters with 32 P-labelled pC7-1 (cDNA corresponding to *cnj C* gene). The standards are a combination of HindIII digested lambda and Hae III digested ϕ X174 RF DNA.

were used (e.g. Taq I, figure 4B). The restriction maps of the genomic DNAs and the cDNA are shown in figure 5. There is an EcoRI site (E') that is somehow modified in the macronucleus so that it is no longer available for cutting; this site is cut by EcoRI in both the micronuclear DNA and the cDNA. We have sequenced this region and confirmed that the restriction sites are as shown. This macronuclear modification is complete (the smaller band is not seen in the macronucleus), site-specific (surrounding EcoRI sites are not modified) and may occur during every macronuclear differen-

Figure 5. Restriction Map Summarizing the Structure of *cnj C*: See figure 2 legend. The cDNA plasmid pC7-1 was used. The numbers under the genomic DNA represent the size in kilobases of the fragments above them. E: EcoRI, E': EcoRI site resistant to digestion in the macronucleus, H: HindIII, h: Hha I, P: Pst I, T: Taq I.

tiation (the modification was seen in strains CU359, CU383, and CU401). We believe that this modification is an adenine methylation (see discussion).

DISCUSSION

All eight genes examined were found to be present in both the somatic (macro-) and germinal (micro-) nuclei. Since the macronucleus is the only transcriptionally active nucleus during most of the life cycle of *Tetrahymena*, genes active at these times must be in both nuclei. During meiotic prophase, however, the micronucleus also appears to be transcribed, peaking at stages II and III of micronuclear elongation (8, 9). Since 10% to 20% of the micronuclear DNA sequence-complexity is omitted from the macronucleus (3), the suggestion has been made (14) that some of this eliminated DNA could contain genes active only during the time when the micronucleus is active; some meiosis-associated genes might be eliminated from the somatic nucleus. The mRNAs from the three conjugation-specific genes were found only during early conjugation (meiotic prophase) and in greatest abundance at the time of micronuclear transcription as would be predicted for micronuclear-specific genes (9). However, all three of these genes were found in both nuclei with no observable DNA rearrangements occurring within or nearby them. These results do not preclude active transcription of these genes only in the micronucleus or in the micronucleus in concert with their macronuclear copies. Genes present only in the micronucleus and transcribed only during meiotic prophase may exist, but their RNA may be much less abundant than the RNA of genes transcribed by the polyploid macronucleus, and therefore they would be less likely to be represented in a cDNA library.

There was no detectable sequence rearrangement in or near the non-

repetitive genes (seven of the eight genes examined). Since the macronucleus is polyploid (45C, see 14), it is theoretically possible that one of the macronuclear copies of a particular gene is rearranged or associated with rearranged sequences and only this copy is transcribed; this type of arrangement probably would not be detected with the resolution of the techniques used. However, previous studies have shown that all *Tetrahymena* genes that have been tested undergo phenotypic assortment in a manner consistent with all or almost all macronuclear copies being functional (15). A rough calculation of the span of unrearranged DNA surrounding the seven non-repetitive genes was made by examining the restriction digest data obtained from side by side comparisons of Southern blots of micronuclear and macronuclear DNA cut with various restriction enzymes and hybridized with the different cDNAs. When restriction fragments that were less than 10 kb were considered (minor differences between restriction fragments larger than 10 kb are difficult to detect), a total span of at least 50 kb surrounding these genes was found to be unrearranged between the two nuclei (18 kb of which is transcribed into mRNA); when restriction fragments as large as 15 kb were considered, the estimated size of unrearranged DNA was at least 75 kb. Thus there is an average of 7 kb (or 11 kb) of unrearranged DNA for each of the seven genes. It has been estimated (4) that approximately 33 kb of DNA separates sites of rearrangement where DNA elimination occurs by breakage and rejoining of chromosomes. Our results would suggest that this elimination is not random with respect to the genes near which it occurs; if it was, we would have expected some of the seven genes to have been associated with rearranged sequences, and not all of the copies of the repetitive gene (pC6) to have been associated with rearranged DNA (see below). There are two published reports where the DNA surrounding protein coding sequences have been examined in *Tetrahymena*; neither of two types of histone H4 genes are associated with rearranged DNA during macronuclear development (5) while there are sequence rearrangements flanking the α -tubulin gene (6). The phenomenon seen in *Oxytricha*, a hypotrich, where intron-like sequences within a gene in the micronucleus are removed from macronuclear DNA (16) has not yet been observed in *Tetrahymena*.

As mentioned above, it appears that each of the five to seven regions of DNA hybridizing to pC6 are rearranged during macronuclear development; this was concluded because each of the EcoRI fragments (smallest of which is 3100 bp) hybridizing to pC6 (755 bp) were of different sizes in the two nuclei. Differences in restriction-fragment sizes between the two nuclei

were also seen when the enzymes HindIII and Hha I were used. These differences are unlikely to be due to methylation differences in the restriction site sequences of Hha I (5'...G-C-G-C...3') and HindIII (5'...A-A-G-C-C-T...3') since cytosine methylation is undetectable in *Tetrahymena* (17) and an adenine must be 5' to a thymidine if it is to be methylated by the *Tetrahymena* DNA adenine methylase (18). Only one mRNA (1470 bases) was detected on RNA blots when pC6 was used as a probe, and all pC6 hybridizing DNA sequences appear to reside on micronuclear chromosome 4. It is not known whether the mRNA hybridizing to pC6 is transcribed by one or all of the pC6 copies. It may be that multiple copies of this gene are necessary because an abundant amount of this mRNA is synthesized during vegetative growth (7).

Differences between the restriction maps of the cDNA contained in pC2-1 and the macro- or micronuclear DNA hybridizing to pC2-1 (figures 2 and 3) suggest that introns are present within the cnj B gene. The correlation between the genomic DNAs and cDNA was confirmed by cross-hybridizing isolated genomic DNA restriction fragments with various cDNA restriction fragments (one such experiment is shown in figure 3). At least six introns appear to be present in the cnj B gene and more probably exist since the cDNA present in pC2-1 (3.6 kb) represents less than one-half of the mRNA (7.4 kb). With the introns included, the size of the cnj B gene is expected to be well over 10 kb. To our knowledge, this is the first evidence reported for introns in a structural gene in ciliates other than the self-splicing intron that has been found in the ribosomal genes of *Tetrahymena* (19). Introns have been found in the non-ribosomal genes of non-ciliated protozoans (eg 20).

A site-specific modification was seen in the macronuclear copy of the cnj C gene (figure 4) making an EcoRI restriction site resistant to cleavage by EcoRI. This modification was complete, site-specific (surrounding EcoRI sites were not modified), and probably occurs during every macronuclear differentiation since this modification was observed in three different strains of *Tetrahymena*. Methylation occurs at the N⁶ position of adenine to 0.8 mol % in the macronucleus; the micronucleus is not methylated (21). The *Tetrahymena* DNA adenine methylase is able to methylate adenine *in vivo* only if thymidine is the next base 3' (18). This requirement is met by the internal adenine of the EcoRI restriction sequence (5'...G-A-A-T-T-C...3') and EcoRI will not cleave at this sequence if this internal adenine is methylated. These facts lead us to believe that the modification occurring

in *cnj C* is an adenine methylation. It is not known what factors are necessary for a particular adenine to be methylated; since several nearby *EcoRI* sequences are not methylated, the *EcoRI* sequence alone is clearly not sufficient. This is the first instance of evidence for site-specific adenine methylation within a eukaryotic gene coding for an mRNA of which we are aware; there are numerous instances of cytosine methylation (22). Although it is not clear what the function of methylation in eukaryotes is, it is known that it affects DNA-protein interactions and there are numerous examples of the demethylation of cytosine being correlated with gene activation (22). Possible roles of methylation in *Tetrahymena* have been discussed recently (23). Since *cnj C* is expressed only during meiotic prophase, it will be interesting to see whether the *EcoRI* site is still modified at this time.

ACKNOWLEDGEMENTS

This work was supported by Grant A3032 from the Natural Sciences and Engineering Research Council of Canada to DWM and Grant GM27871 from the National Institute of Health to PJB.

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