Complex endonucleolytic cleavage pattern during early events in the processing of pre-rRNA in the lower eukaryote, *Tetrahymena thermophila*

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ABSTRACT

We have analysed nuclear RNA from T. thermophila by RNA transfer hybridization using cloned rDNA fragments. A very high number of in vivo intermediates and by-products of rRNA processing were identified. These include putative intermediates of the splicing process and alternative products resulting from temporal variability in various endonucleolytic cleavages. In addition, four small RNA species including only transcribed spacer sequences were detected. These are (1) the IVS RNA (∿ 400 bases), the by-product of the splicing process, (2) a fragment from the internal transcribed spacer (∿ 360 bases), possibly resulting from 3'-end processing of pre-17S rRNA, (3) a fragment comprising most or all of the external transcribed spacer (∿ 600 bases) obviously representing the major by-product of 5'-end processing, and, in addition, (4) a small fragment from the initiation region (∿ 230 bases) which might be a product of premature transcription termination.

INTRODUCTION

The ciliated protozoa of the genus Tetrahymena contain multiple extrachromosomal rDNA molecules of palindromic structure in the vegetative macronucleus (1,2), and show very high rates of pre-rRNA synthesis and ribosome formation during optimal growth (3,4; for review see 5,6). In some species, including T. thermophila, the 26S rRNA coding regions on these amplified rDNA molecules are interrupted by an intervening sequence (7,8). We have recently performed transcription mapping of the rDNA of T. thermophila by Southern, S_1 nuclease, and R-loop analyses (9). From these studies the basic features of gene organization and pre-rRNA processing were deduced (summarized in Fig. 1): A certain portion of the molecules in the pre-rRNA fraction are colinear with the entire gene region and include the transcribed intervening sequence (10,11). Excision of the IVS RNA and splicing of flanking exon sequences is a very early processing step (step (1), see Fig. 1; reviewed in 6), obviously preceeding the following major events. These are (2) the cleavage of the pre-rRNA into pre-17S and pre-26S rRNA, (3) the removal of the external transcribed spacer (ETS) segment, (4) the removal of the

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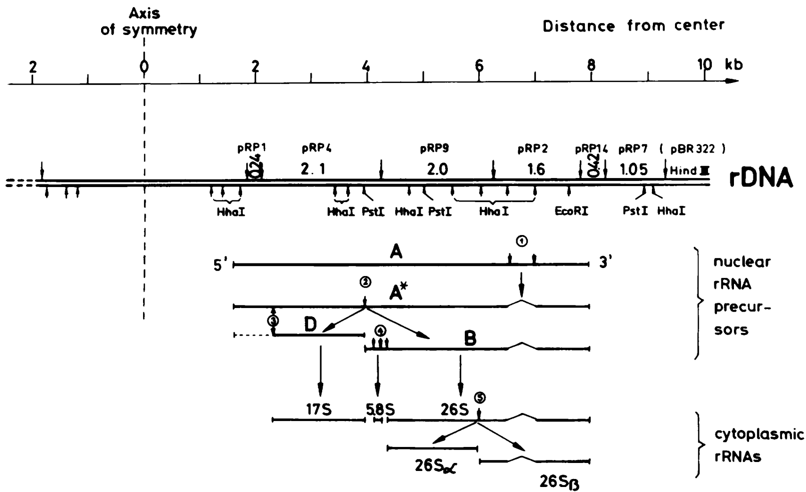

Fig. 1 Transcription map of extrachromosomal rDNA from Tetrahymena thermophila
Only one half of the palindromic rDNA is shown. Some of the restriction sites used in recent mapping studies (9) are included. The approximate sizes of cloned HindIII fragments and the code numbers of the respective recombinant plasmids are indicated above the rDNA axis. Below the rDNA the coding regions of abundant rRNA species mapped recently (9) are drawn and arranged from top to bottom roughly in the order of their appearance during pre-rRNA processing (A, unspliced and A*, spliced pre-rRNA; B, pre-26S rRNA; D, pre-17S rRNA). Putative sites of main processing events are indicated by small arrows and numbered in circles (for details see INTRODUCTION).

internal transcribed spacer (ITS) regions including the formation of 5.8S rRNA, and (5) the introduction of a central hidden break into the 26S rRNA. Putative transcription initiation and termination sites have been determined at the sequence level, and the removal of a small 3'-extension from pre-rRNA has been reported (12-16).

Except for the specialities represented by pre-rRNA splicing and nicking of 26S rRNA, pre-rRNA processing in Tetrahymena resembles the general scheme for eukaryotes (for a recent review see 17). It has been assumed, however, that in contrast to higher eukaryotes, where a certain degree of flexibility in the temporal order of endonucleolytic cleavages has been observed (18-21), a simpler and more rigid sequence of processing cuts probably occurs in lower eukaryotes (cf. 17). This concept has been supported by findings in yeast and included in a recent model by Planta and collaborators (22), which suggest that the pre-rRNA processing in these cells proceeds in a strict order of successive pairs of simultaneous cuts.

Previous studies with Tetrahymena have already indicated that some flexibility in the order of pre-rRNA processing events 2 and 3 (see above) may exist in this organism (9,23). However, with the methods used(see above), only relatively abundant rRNA precursor molecules and species of distinctly different lengths could be identified. In this study, we have used RNA gel transfer and blot hybridization to resolve possible alternative intermediates and to detect and characterize small and infrequent RNA species.

Specifically, we were interested in (i) the number, position, and order of endonucleolytic cleavages involved in the different processing events, (ii) the characterization of short-lived processing intermediates, and (iii) the existence of free transcribed spacer sequences removed by the endonucleolytic cleavages.

The results suggest that the pattern of endonucleolytic cleavages during pre-rRNA processing in T. thermophila is highly complex and exhibits considerable flexibility in the temporal order of various steps.

MATERIALS AND METHODS

Cell culture and cell fractionation

Tetrahymena thermophila cultures were grown to mid-log growth phase in a complex organic medium (24). The cells were fractionated and macronuclei isolated as described (4,25).

Extraction and separation of RNA

Total RNA was extracted from isolated macronuclei (26) and separated in 1.5% agarose slab gels (14x10x0.3 cm) submersed in buffer. RNA samples (10-30 μg) were either denatured by glyoxalation before electrophoresis in a sodium phosphate buffer system (27), or run in native form in 50 mM boric acid, 5 mM sodium tetraborate, 10 mM sodium sulfate, 1 mM EDTA, pH 8.0. After electrophoresis gels were treated and stained with ethidium bromide as described by Alwine et al. (27), and photographed under UV transillumination with a Polaroid PM 2 camera.

RNA transfer to diazotized paper and blot hybridization

Diazobenzyloxymethyl(DBM) paper was prepared, RNA transferred from the gels onto the paper, and the blots preincubated essentially as described by Alwine et al. (27). Hybridization was at 40°C for 18-24 hr in 50% formamide, 0.9% NaCl, 50 mM sodium phosphate, 5 mM EDTA, 0.1% SDS, and 0.02% each of bovine serum albumin, ficoll 400 and polyvinylpyrrolidone. The hybridization mixture contained 300 μg/ml sheared and denatured calf thymus DNA, and the ^{32}P-labelled DNA probes (see below) were added to about 40 ng/ml.

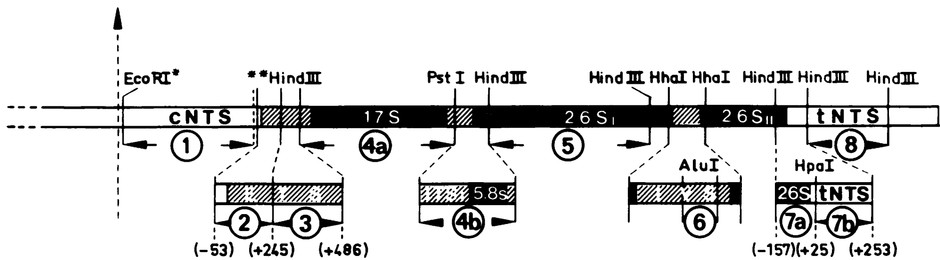

Fig. 2 Gene map of rDNA and location of restriction fragments used as hybridization probes
The presentation of rDNA is similar to that shown in Fig. 1. Specific restriction fragments are drawn in an enlarged scale (x3) below. The various regions from the symmetry center towards the free termini (distal ends) represent: cNTS, the central nontranscribed spacer; ETS, the external transcribed spacer; 17S, the 17S rRNA coding region; ITS, the internal transcribed spacer including the 5.8S rRNA coding region; $26S_I$ and $26S_{II}$, the 26S rRNA coding regions interrupted by the intervening sequence (IVS); tNTS, the terminal nontranscribed spacer. The plasmids or derived subfragments used as hybridization probes for the various regions are numbered consecutively: (1) pGY 19, (2) pJE 298, (3) pRP 1, (4a) proximal and (4b) distal PstI subfragment of the insert in pRP 4, (5) pRP 9, (6) pJE 41, (7a) proximal and (7b) distal HpaI subfragment of the insert in pRP 14, (8) pRP 7.

Papers were washed (cf. 27), dried, and exposed to Kodak XAR-5 film at -80°C using Kodak intensifying screens. For repeated hybridization of RNA blots with different probes, bound DNA was removed by washing three times in 99% formamide at 65°C and subsequently the paper was preincubated again.

Densitometry of autoradiograms and of negatives from stained gels was performed at 480 nm using a scanning accessory of a Zeiss PMQ III photometer. Relative areas under the peaks were determined by cutting out and weighing.

Tetrahymena rDNA containing plasmids and derived subfragments used as hybridization probes

Cloned rDNA fragments that span the entire pre-rRNA transcription unit and adjacent spacer regions were used. Most of the central non transcribed spacer (cNTS) is included in pGY 19 (28), which was kindly provided by R.E. Pearlman (Toronto). The plasmid consists of a pACYC 184 vector with an inserted partial EcoRI*-fragment which begins at about 40 bp from the center of the palindromic rDNA and has its distal end somewhere between 120-200 bp upstream from the transcription initiation site (see Fig. 2, probe 1). The regions around the putative major *in vivo* initiation site (position -53 to +245; 16) is contained in pJE 298. It consists of a

298 bp EcoRI*-HindIII fragment (cf. probe 2 in Fig. 2) inserted within the EcoRI and HindIII sites of pBR 322, and was obtained from J. Engberg (Copenhagen). Most of the following hybridization probes were based on HindIII fragments previously cloned into pBR 322 (ref. 9 and Fig. 1). The insert in pRP 1 is a 240 bp fragment from the middle (position +246 to +486) of the 650 bp external transcribed spacer (probe 3 in Fig. 2). The adjacent HindIII fragment (∿ 2.1 kb) is contained in pRP 4 and includes the distal segment of the ETS region (pos. +487 to +650), the entire 17S rRNA coding region, and the major part of the internal transcribed spacer (ITS), including the 5.8S coding region. The ITS was separated from the 17S rRNA coding region by cutting the insert with PstI (9; see subfragments labelled 4a and 4b in Figure 2). The 5.8S coding region was placed near the distal end of the 0.4 kb subfragment (4b), using a BglII site predicted from the 5.8S rRNA sequence at around position 100 (29). The HindIII fragment in pRP 9 (about 2 kb; probe 5 in Fig. 2) includes the promotor-proximal half of the 26S rRNA coding region ($26S_I$). The adjacent HindIII fragment (1620 bp) includes the intervening sequence (9), and is contained in pRP2. A 484 bp subfragment, including the 413 bp IVS (cf. 30), is drawn in an enlarged scale in Fig. 2. As an intron specific probe a 145 bp AluI fragment (probe 6, Fig. 2) of the T. pigmentosa rDNA intron was used (the respective region is over 90% homologous between both Tetrahymena species; cf. 30,31). This fragment was originally inserted into the BamHI site of pBR 313 (plasmid pTpAA 1; kindly provided by M. Wild, Yale) and has since been transferred to pBR 322 by J. Engberg (plasmid pJE 41). The insert in pRP 14 (415 bp) includes the putative termination site of pre-rRNA transcription and the 3'-terminal coding region of 26S rRNA (14). By cutting with HpaI at 25 bp downstream from the mapped termination site, two subfragments were obtained specific, respectively, for the 3'-terminal coding region, and the sequences immediately downstream from the putative termination site (probes 7a and 7b in Fig. 2). The insert in pRP 7 (about 1 kb; probe 8, Fig. 2) is from the terminal nontranscribed spacer.

Isolation and in vitro labelling of plasmid DNA and derived subfragments

E. coli cells (HB 101, JF 1161, and NF 1513) carrying the different plasmids were grown to mid-log phase, the plasmids amplified by chloramphenicol or spectinomycin treatment, and the plasmid DNA isolated by the cleared lysate method essentially as described by Clewell & Helinski (32). After digestion of the plasmid DNA with the respective restriction enzymes under conditions suggested by the suppliers (BRL and Boehringer), subfrag-

ments were separated using 1-2% low melting point agarose (BRL). Samples were separated in gels containing 20 mM sodium acetate, 2 mM EDTA, 30 mM Tris/HCl, pH 7.8. After staining with ethidium bromide bands were cut out from the gels, and the DNA recovered by extraction with hot phenol and precipitation with ethanol (33).

For hybridization, the DNA probes were labelled with ^{32}P (α-dTTP, 400 Ci/mmol, Amersham) _in vitro_ by nick-translation using a method modified from Rigby et al. (34). The specific radioactivities usually ranged between 5 to 10 x 10^7 cpm/μg DNA. Prior to use the DNA was denatured by boiling in 10 mM Tris/HCl, pH 7.2 and rapid chilling on ice.

RESULTS

Electrophoretic separation and blot hybridization of total nuclear RNA

RNA from _Tetrahymena_ macronuclei can be fractionated into 4 major high molecular weight fractions which contain rRNA precursor molecules (designated A-D; 9,10,26). When the nuclear RNA was denatured by glyoxalation and separated in 1.5% agarose gels, at least 8 discrete bands appeared in the upper region of the gel (Fig. 3a, lane T). The relatively broad band with the lowest mobility (corresponding to molecules of an average size of 6.3 to 6.4 kilobases, kb) includes the pre-rRNA molecules (fraction A), and is actually a mixture of at least 3 different size classes which was not readily resolved under these separation conditions (see below). Fraction B, comprising the pre-26S rRNA molecules (cf. 9), was resolved into two species with sizes of approximately 3.8 kb (B_1) and 3.5 kb (B_2). Fraction D (17S region) was also resolved into two closely spaced bands, corresponding to molecular sizes of 1.8 kb (D_1) and 1.65 kb (D_2). Between the B and D species (C region) 3 minor bands could be revealed corresponding to molecules of approximately 2.9 kb (C_1), 2.5 kb (C_2), and 2.2 kb (C_3) in length. In order to determine the sequence content of these putative rRNA precursor molecules, and to identify additional rDNA transcripts not visible in the fluorescence pattern, the RNA was transferred from the gel to DBM paper. The respective RNA-blot was then hybridized to a variety of rDNA probes spanning the entire rRNA transcription unit and flanking spacer regions (cf. MATERIALS AND METHODS and Fig. 2).

In Fig. 3b the hybridization patterns obtained with probes specific for the cNTS and the ETS including the transcription initiation region are presented. The absence of detectable hybridization with pGY 19 (Fig. 3b, lane 1) showed that nuclear RNA synthesized _in vivo_ contained no sequences comple-

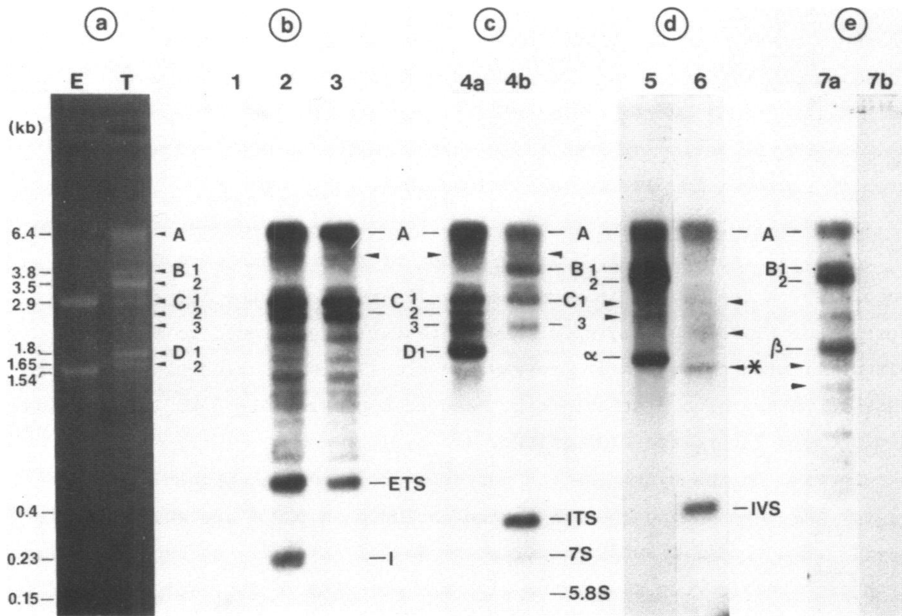

Fig. 3 Blot hybridization of macronuclear RNA with various rDNA probes
RNA extracted from isolated macronuclei was denatured by glyoxalation, subjected to electrophoresis in 1.5% agarose gels, and blotted to DBM paper. The blot was then successively hybridized to various nick-translated rDNA probes. (a) Ethidium bromide stained gel. The lane labelled T documents the separation of the *Tetrahymena* RNA before blotting. Approximate sizes of transcripts were determined with *E.coli* rRNAs as standards (displayed in lane E), as well as using defined *Tetrahymena* RNA species detected in the autoradiograms. For hybridization of the blot, probes were used from the cNTS and the ETS including the transcription initiation region (b), from the 17S rRNA coding region and the ITS (c), from the 5'-half of the 26S rRNA coding region and the IVS (d), and from the transcription termination region (e). The individual lanes are labelled with the number of the respective probe used, as indicated in Fig. 2. For designation of specific bands see text.

mentary to the cNTS region beginning at 120-200 bp upstream from the putative initiation site. With the plasmid pJE 298 including the sequences around the mapped initiation site (Fig. 3b, lane 2), and the ETS-specific probe (pRP 1, Fig. 3b, lane 3) almost identical hybridization patterns were produced, except for one strong band in the low molecular weight region (see below). In the upper (high mol wt) region, strong hybridization signals were apparently associated with the pre-rRNA fraction (A), and two putative pre-17S rRNA species (C_1 and C_2, see below). Another major band in both patterns corresponds to an RNA species of approximately 600 bases which

most likely represents a major part of the ETS removed during pre-rRNA processing. It was thus designated ETS fragment. With pJE 298 (lane 2) an additional strong band at about 230 bases appeared (initiation fragment, I). The similar strong hybridization signals suggest that both fragments (ETS and I) seem to overlap substantially in the region between -53 and +245 which is included in pJE 298 (see DISCUSSION).

In addition to the described major hybridization signals, in the autoradiograms in lane 2 and 3, a large number of faint bands were detected, especially in the region between the pre-17S rRNAs and the ETS fragment. These minor ETS-containing species were tentatively assumed to be the result of limited cleavages of pre-rRNA or pre-17S rRNA during isolation. The possible nature of a distinct faint band at approximately 5 kb (arrows) will be discussed later (see DISCUSSION).

In Fig. 3c the hybridization patterns produced with probes specific for the 17S rRNA coding and ITS region included in pRP 4 are shown. With the 17S rRNA-specific subfragment (probe 4a; cf. Fig. 2), as expected, the strongest signals were associated with the pre-rRNA region, and with the 17S rRNA or its immediate nuclear precursor (included in the 1.8 kb band D_1, Fig. 3c, lane 4a; cf. also 9,26). In between these signals, 3 discrete, but somewhat less intensive bands at 2.9, 2.5 and 2.2 kb were present. The 2.9 kb (C_1), and the 2.5 kb (C_2) species represent those pre-17S rRNA molecules, including most or all of the ETS sequences described above. The structure of the third putative pre-17S rRNA species (C_3, 2.2 kb) is explained below. With probe 4b containing only sequences from the ITS (cf. Fig. 2) major signals were associated with the pre-rRNA (A), the 3.8 kb pre-26S species (B_1), and the 2.9 kb (C_1) and 2.2 kb (C_3, pre-17S rRNA species. These large molecules thus include significant stretches of ITS sequences. In addition, a small RNA (about 360 bases) was heavily labelled. This species was also present in RNA samples separated under non-denaturing conditions (not shown), and was therefore assumed to be a free transcribed spacer segment and designated ITS fragment. The additional faint bands at about 230 and 150 bases are only present under denaturing conditions, and thus most likely represent the 5.8S rRNA and a putative larger precursor (7S) (see DISCUSSION).

In the next two lanes (Fig. 3d), the blot was hybridized to probes specific for the 5'-half of 26S rRNA and to the IVS region, respectively. With pRP 9 (probe 5, cf. Fig. 2) the pre-rRNA and the two pre-26S rRNA species (B_1 and B_2) produced strong signals. The strong band at 1.65 kb (D_2) in-

dicates the presence of substantial amounts of 26S rRNA α-fragments in the nuclear RNA (see DISCUSSION). Two faint bands at about 2.8 kb and 2.4 kb (arrowheads) were also revealed. The clear signal associated with the pre-rRNA region after hybridization with the IVS-specific probe (pJE 41, Fig. 3d, lane 6) indicates the presence of substantial amounts of unspliced molecules in this fraction (see below and 8,9). The strong band in the low mol wt region includes the IVS RNA, the by-product excised from pre-RNA during splicing (6,35,36). A minor, but distinct band could be recognized at about 1.5 kb (arrowhead with asterisk). This species also includes 3'-exon sequences (see Fig. 3e, lane 7a, arrowhead). A similar species was also found by Brehm & Cech (37) and may be an intermediate of the splicing process (see DISCUSSION). Two additional faint bands including IVS sequences at about 2.8 and 2.2 kb were also detected. The former one was at the same position as the respective faint band in lane 5.

In Fig. 3e the hybridization patterns obtained with probes from the transcription termination region included in pRP 14 are shown. The proximal 180 bp HpaI-subfragment (probe 7a, cf. Fig. 2) contains the mapped transcription termination site (at pos. 158) and the 3'-terminal end of the 26S rRNA coding region (at pos. 143; 14). Using this fragment, strong hybridization signals were associated with pre-rRNA, the pre-26S rRNA species (B_1 and B_2), as well as the 26S rRNA ß-fragment (1.8 kb). A lot of minor bands were also apparent especially in the region below the ß-fragment. The origin of most of these fragments may be similar to those observed with probes from the initiation region. Distinct bands at about 1.5 kb and 1.1 kb (arrows) may correspond to the $26S_{II}$ (3'-exon) segment with and without the IVS region. No hybridization could be detected with the distal HpaI subfragment of the insert in pRP 14 (probe 7b) beginning 25 bp downstream from the mapped termination site. The same was true, even after extended exposure times, with pRP 7 (probe 8, not shown), indicating that no transcripts significantly extended through the mapped termination site.

Analysis of differences in the sequence content of pre-rRNA subspecies

As shown and mentioned above, it was usually not possible, with glyoxalated RNA samples, to separate the different size classes contained in the pre-rRNA fraction. In Fig. 4, therefore, undenatured RNA samples were separated in borate buffer. As can be seen in Fig. 4a, lane 1, with RNA from isolated macronuclei, the pre-rRNA fraction could be resolved under these conditions into at least 3 subfractions including molecules of about 5.8 kb (A_3), 6.4 kb (A_2), and up to approximately 6.8 kb (A_1). A densitometric scan

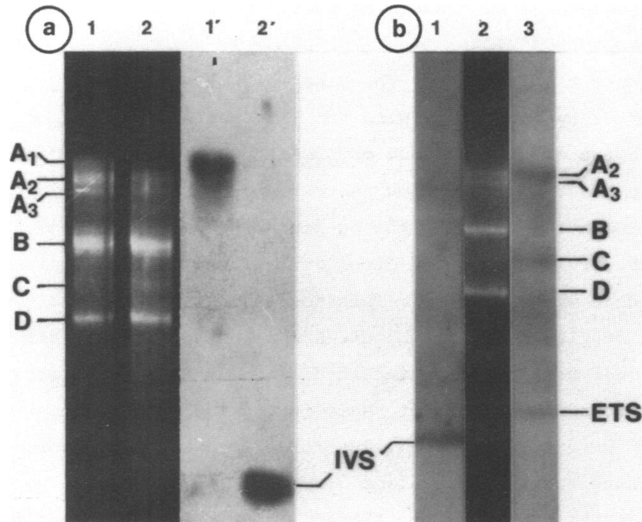

Fig. 4 Analysis of the differences in sequence content of pre-rRNA subfractions
RNA was extracted from (a) isolated macronuclei before (lane 1) and after (lane 2) in vitro incubation under "processing conditions" (38) for 15 min, as well as (b) from isolated large RNP particles (39). The RNA was separated in native form in borate buffer in 1.5% agarose gels and blotted to DBM paper. Hybridization of the nuclear RNA (a) was with the IVS-specific probe (pJE 41; lanes 1' and 2'), and of the particle RNA (b) with the IVS-specific (lane 1) and ETS-specific probe (pRP 1, lane 3).

of the negative showed that these subfractions include around 15% (A_1), 60% (A_2), and 25% (A_3) of total material in the pre-rRNA region. Three subfractions were also revealed in the B region, but only one sharp band in the D region. When the RNA was transferred to DBM paper and hybridized to the IVS-specific probe (pJE 41), the molecules with the lowest mobility (A_1) preferentially hybridized (lane 1'), and thus contained unspliced pre-rRNA molecules. The light smear in the hybridization signal extending down to a region corresponding to about 5 kb may be largely due to growing pre-rRNA chains already including the total or parts of the IVS region. When a parallel sample of macronuclei was incubated in vitro under "processing conditions" (38) for 15 min, and the RNA then extracted and analysed, the A_1 region disappeared in the fluorescence pattern (lane 2), as did the hybridization signal in this region (lane 2'). This signal was, however, replaced by a strong band in the region of the free IVS RNA. This indicates that the in vivo synthesized primary transcripts of rRNA had been spliced under these conditions, and that this event is correlated with the disappearance of the

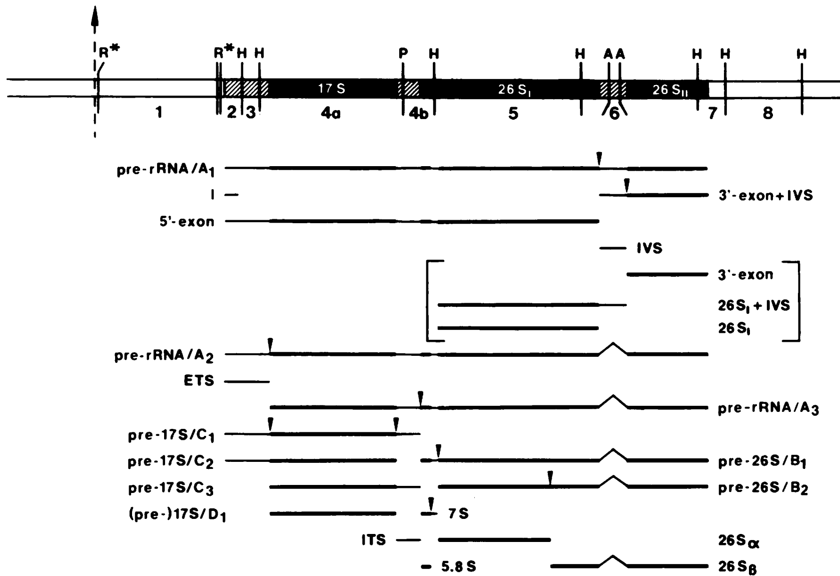

Fig. 5 Map of various rDNA transcripts identified in nuclear RNA of T. thermophila
The presentation of rDNA structure on the top is similar as in Fig. 2, including the positions of the probes (1-8) used in this study. The coding regions of different transcripts, as deduced from the hybridization data, are drawn as single lines (thick bars, rRNA sequences; thin lines, transcribed spacer sequences). Small arrows denote the position of possible endonycleolytic cleavages on the different rRNA precursors resulting in the products (and by-products) drawn just below in each case. For details see DISCUSSION.

substrate for the splicing reaction (subfraction A_1) and the appearance of the by-product, the excised IVS RNA.

To explain the main difference in sequence content between subfractions A_2 and A_3 , the results of an analysis of the RNA contained in isolated large (> 80S) RNP-particles (39) is shown (Fig. 4b). The two distinct bands in the pre-RNA region (lane 2) in this case must correspond to A_2 and A_3, since none of them significantly hybridized to the IVS-specific probe (lane 1; see, however, the clear signal associated with the IVS RNA !). In this specific case the pre-RNA was spliced during isolation of the RNP-particles (39). Hybridization with the ETS-specific probe (pRP 1) clearly showed that only the upper band (A_2) contained sequences from the middle of the ETS region. Thus, the A_3 subfraction obviously includes those pre-rRNA molecules which have already lost the segment represented in the ETS fragment (see DISCUSSION).

In Fig. 5 our interpretation of the RNA transfer hybridization data is presented in schematic form and the results will be discussed on the basis of this map in the next section.

DISCUSSION

We have characterized, by RNA transfer hybridization using various rDNA probes, a high number of putative in vivo intermediates and by-products of rRNA processing in Tetrahymena thermophila (cf. Fig. 5). The largest transcripts are included in the pre-rRNA subfraction A_1 and most of them obviously contain the transcribed intron. The proportion of this subfraction (15% of total) is consistent with our previous findings that a substantial portion of full-length transcripts is unspliced (9,10). Some microheterogeneity may exist in this pre-rRNA subfraction (A_1) due to a rapid removal of some 15 bases from the 3'-terminal end (14). Pre-rRNA splicing in Tetrahymena is a very rapid event in vivo (9,10,37), and is an autocatalytic process not requiring 3'-terminal exon sequences at least in vitro (40,41). It is, therefore, possible that a certain portion of pre-rRNA molecules does not go through the A_1 stage, but is spliced before termination. In this case pre-rRNA species A_2 would be the primary finished transcript which otherwise represents the first prominent processing intermediate. The by-product of the endonucleolytic steps involved in splicing, the free IVS RNA (cf. 6,35,36), is moderately stable in vivo and can be found in substantial amounts in the nuclear RNA (cf. also 37). In addition, a number of RNA molecules containing intron sequences linked to specific parts of exon sequences could be detected in the blots. The most prominent and distinct is a 1.5 kb species including the 3'-terminal exon sequences. This molecule may be an intermediate of the splicing process with the first endonucleolytic cut occurring at the 5'-end of the IVS, as proposed by the phosphoester transfer model of Cech et al. (6,36,41). In support of this assumption, current experiments, using ^{32}P-GTP as cofactor (42), have shown that these molecules can be endlabelled during in vitro splicing of pre-rRNA in isolated macronuclei (38). We have also identified an RNA species which from its length (∿ 5 kb) and hybridization characteristics may be the corresponding 5'-exon half of the complex pre-rRNA splicing intermediate. Three other species deserve comment in this context: a 2.8 kb species including the 5'-half of pre-26S and most or all of the IVS segment, a 2.4 kb species only including the 5'-exon sequences of pre-26S rRNA (a similar species was recently mapped and termed "C", cf. 9), and a free 3'-exon segment. These species are

put into square brackets in Fig. 5, because we suspect them to be products of aberrant splice cuts and/or impaired exon ligation. Analog species have also been found as products of (mis)splicing of rRNA in yeast mitochondria (43). We have never observed, either _in vivo_ or _in vitro_, intact pre-26S rRNA with intron sequences, indicating that the normal splicing process is always completed before the cleavage of pre-rRNA into pre-26S and pre-17S rRNA.

From the mapping of the 3'-end of pre-17S rRNA/C_1 and the 5'-end of pre-26S rRNA/B_1 within the internal transcribed spacer, we assume that the central cleavage of the pre-rRNA (processing event 2) is a single endonycleolytic cut at or near the 5'-end of the 5.8S rRNA sequence (cf. similar results with mammalian cells,2). As to the timing of this step with processing event 3 (ETS removal), our data show that about 25% of the pre-rRNA molecules (included in subfraction A_3) have already lost most or all of the ETS segment. This confirms earlier indications that in _Tetrahymena_ processing events 2 and 3 can occur in a variable temporal order (9,23).

The significance of the small RNA species from the ETS region is somewhat puzzling, considering their individual lengths and positions, and the total length of the ETS determined by a presumptive unique initiation site at 650 bp upstream from the 5'-end of the 17S rRNA coding region (16). From its size it is possible that the ETS fragment (600 $\pm$ 50 bases) contains most or all of the ETS segment. Assuming that this species is the major by-product of ETS processing, the nature of the small RNA species (230 bases) from the initiation region (I fragment) is not easily explained. This fragment might be (1) a product of early pre-rRNA processing at the 5'-end, (2) a product of alternative ETS processing, or (3) a product of premature termination (attenuation). The first possibility is unlikely, because from the relative hybridization intensities the I fragment and the ETS fragment seem to substantially overlap in the initiation region. Whether both species originate from the mapped initiation site cannot be determined from the present hybridization data. The second possibility is also unlikely, because in this case a corresponding 3'-subfragment of the ETS of about 400 bases should be present. Such a species was not detected with the ETS-specific probe (pRP1). The same would be true, if this species were a 5'-degradation product of the ETS fragment. An intriguing possibility is that this species represents a promoter proximal short transcript which was never included in longer RNAs (i.e. a product of premature termination, cf. its position in Fig. 5). This notion is also consistent with S_1-mapping studies of Saiga et al. (15), which could not locate any major RNA 5'-end within ap-

proximately 500 bp downstream from the putative initiation site in *T. pyriformis*. It may be that in eukaryotes, as has already been proposed from *in vitro* results for *E. coli* (44), transcription of ribosomal genes is regulated in part through an attenuation mechanism. Studies including isolation and sequence mapping of these small RNA species are underway to clarify these questions.

Hybridization with the ITS-specific probe identified two pre-17S rRNA species (C_1 and C_3) including ITS sequences. Thus, in contrast to the conclusions drawn from our previous mapping studies (9), we have to envisage considerable 3'-end processing of the pre-17S rRNA. We propose that these sequences are removed in a single endonucleolytic step resulting in the ITS fragment as a by-product. The simultaneous existence of pre-17S rRNA species C_2 and C_3 is explained by a temporal variability between ITS and ETS removal. Whether the 1.8 kb species (D_1) is either identical to the mature 17S rRNA or contains some additional sequences has to be determined by sequence analysis.

The processing steps at the 5'-end of pre-26S rRNA/B_1 include at least one endonucleolytic step leading to the separation of the 5.8S rRNA sequence. A putative 7S precursor to 5.8S rRNA was previously separated from an isolated pre-26S rRNA fraction (26). A similar species of 230 bases was identified in this study with the ITS-specific probe. It is drawn in Fig. 5 as comprising the 5.8S sequence plus most or all of the ITS II segment (max. 100 bp in length, cf. 9). Similar 5.8S rRNA precursors have been characterized from other organisms (21,45,46). Determination of the exact structure of the 7S molecule will be performed in conjunction with the exact intracellular localization of the 7S → 5.8S transition. Recent studies have indicated that this step may occur mainly or exclusively in the cytoplasm. The same is true for the last processing event, the central cleavage of the 26S rRNA into the α and β fragment (26). The presence of some 5.8S rRNA and of the large 26S rRNA subfragments in nuclear RNA may be mainly due to cytoplasmic contamination, including the ribosomes still associated with the outer nuclear membrane in our preparations (unpublished observations).

In conclusion, the results suggest that there is considerable variability in the temporal order of early endonucleolytic cleavages following splicing of pre-rRNA in *T. thermophila*. This flexibility in the sequence of various processing events results in a high number of alternative intermediates of rRNA maturation. Clearly, in this respect *Tetrahymena* resembles higher animals (cf. 20,21; for review see 17) more than yeast (22). The physiolo-

gical significance of the simultaneous existence of multiple rRNA processing pathways awaits clarification.

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