In vitro splicing of the ribosomal RNA precursor in isolated nucleoli from Tetrahymena

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ABSTRACT

    The macronuclear rRNA genes of Tetrahymena thermophila con-
tain an 0.4 kb intervening sequence in the 26S rRNA coding re-
gion. The sequence is represented within the primary transcrip-
tion product. We demonstrate in this paper that the enzyme activ-
ities necessary for the endonucleolytic cleavage as well as for
the ligation of the transcript are associated with the isolated
nucleoli. Both of these processes occur posttranscriptionally.
The intervening sequence is excised as an unique molecule, which
is stable in vitro. About 50% of the in vitro synthesized RNA is
processed. Faithful in vitro transcription occurs in the pres-
ence of the divalent ions Mg^{2+}, Mn^{2+} and Co^{2+} while processing
takes place only in the presence of Mg^{2+}. The absolute require-
ment for Mg^{2+} in the excision reaction enables us to synthesize
labelled pre-rRNA in the presence of Mn^{2+} or Co^{2+}. The synthe-
sized RNA can be used as a substrate in studies of the process-
ing enzymes in vitro.

INTRODUCTION

    In Tetrahymena the genes coding for the ribosomal RNA is

present in multiple copies located on linear extrachromosomal

rDNA molecules[1]. Each rDNA molecule is a giant palindrome con-

taining two cistrons for the pre-35S rRNA[2,3]. Recently, several

investigators have observed that the rDNA from several Tetrahy-

mena species and strains are interrupted in the 26S rRNA coding

region by an intervening sequence of 400 bp[4-9]. As all of the

macronuclear rDNA molecules of Tetrahymena thermophila contain

the intervening sequence, it is evident that these genes are

functional in vivo. In addition, in vivo studies have demon-

strated that the intervening sequence is contained within the

primary transcription product of the gene and that the posttran-

scriptional removal of the intervening RNA sequence is a very

early processing event in this organism[5]. Similar observations have been made for the rRNA genes in Physarum[10]. In contrast, those of the rRNA genes in Drosophila melanogaster containing an insertion do not contribute significantly to the production of 28S rRNA[11,12].

In order to study the regulation of the rRNA genes in Tetrahymena we have developed techniques to isolate transcriptionally active nucleoli[13,14]. The transcriptional properties of the nucleoli mimic the in vivo process with respect to strand selection, elongation rate and termination properties[13-15]. The current studies were undertaken to investigate if processing of the transcribed intervening sequence would occur in isolated nucleoli. The data presented show that the nucleolar system is able to perform both the endonucleolytic cleavage and ligation around the intervening sequence in the primary transcript. The intervening sequence is excised from the pre-rRNA as an unique molecule. In addition, methods have been developed to control the processing event in vitro. The results are in accordance with recent data of Zaug and Cech[8], which demonstrate that isolated macronuclei of Tetrahymena are able to excise the intervening sequence from pre-35S rRNA.

MATERIALS AND METHODS

Culture conditions. Tetrahymena thermophila (strain B 1868-7) was grown in a complex medium to a density of 60,000-80,000 cells per ml as previously described[13,14].

Isolation of nucleoli containing transcriptionally active r-chromatin. Cells were harvested by centrifugation at 300xg and washed with 30 volumes of nuclei buffer (0.1 M sucrose, 10 mM Tris-HCl pH 7.2, 3 mM $CaCl_2$, 1 mM $MgCl_2$) at 4 °C. The cells were lysed with nuclei buffer containing 0.3% Nonidet P40 and the macronuclei were collected by centrifugation at 500xg. The macronuclei were washed once with nuclei buffer containing 0.1% Nonidet P40 and once with nuclei buffer and then resuspended in 10 volumes of extraction buffer (10 mM Tris-HCl, pH 7.2, 120 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1 mM 2-mercaptoethanol) and homogenized gently in a glass homogenizer. The extracted nuclei were pelleted by centrifugation at 500 xg. The supernatant containing

the nucleoli was layered on top of a sucrose cushion (1.5 ml 80% sucrose and 3.5 ml 50% sucrose in 10 mM Tris-HCl pH 7.2, 120 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol). After 30 min of centrifugation at 15,000xg, a volume of 3 ml containing the nucleoli was collected from the bottom of the tube[12]

Isolation of rDNA. The rDNA was isolated from whole Tetrahymena cells in its native palindromic form by the method described by Din and Engberg[16] except that the CsCl density gradient centrifugation was performed in the presence of 1 µg of Hoechst dye (Hoechst No. 33258) per µg of DNA.

In vitro transcription and processing of rRNA. Nucleoli were incubated in the presence of 4.9 mM $(NH_4)_2SO_4$, 5.0 mM $MgCl_2$, 3.8 mM 2-mercaptoethanol, 0.50 mM EDTA, 32 mM Tris-HCl pH 7.2, 2.1 mM KCl, 57 mM NaCl, 95 µM ATP, CTP and GTP and 4.2 µM of $\alpha^{32}P$-UTP for 30 min at 25 °C unless otherwise indicated. To prevent processing, $MgCl_2$ was replaced either by 0.25 mM $MnCl_2$ or 0.15 mM $CoCl_2$. Incubation was stopped with 0.5% SDS and 4.2 M urea. After heating of the samples to 45 °C, the RNA products were analysed in the presence of markers on 1.8% or 3% agarose gels containing 5 M urea[17].

Southern hybridization. rDNA was digested with BglII+HhaI restriction endonucleases and applied on 4% agarose gels. After ethidium bromide staining, the gel was UV irradiated for 30 min. The DNA was then denatured and blotted onto nitrocellulose filters (Millipore) as described by Southern[18]. rRNA was synthesized and electrophoresed as mentioned above. In vitro synthesized total rRNA and 0.4 kb RNA were extracted from the gels. The hybridization occured in the presence of 2 × SSC, 10 mM HEPES pH 7.0, 0.5% SDS and 40% formamide at 41 °C for 2 days. After hybridization the strips were washed in 6 × SSC containing 1 mM pyrophosphate. Autoradiography was carried out at -70 °C.

R-Loop formation. Nucleic acids from isolated nucleoli were extracted with phenol–chloroform-isoamylalcohol and precipitated by ethanol. The pellet containing both endogenous rDNA and rRNA was dissolved in 20 mM Tris-HCl pH 7.4 made to 70% formamide, 100 mM NaCl, 10 mM EDTA , and 100 mM HEPES pH 7.8 and incubated at 70°C for 15 min in order to denature the rDNA. Hybridization was performed by incubation at 41°C for 16-18 h[5]. The R-loops

were stabilized by fixation with glyoxal for 2 h at 13°C accord-
ing to the method by Kabach et al.[19]. The hybridization mixture
was diluted into 50% formamide, 100 mM Tris-HCl pH 7.4, 100 mM
NaCl, 10 mM EDTA. The solution was spread in the presence of
0.1 mg/ml of cytochrome C on a hypophase of 10-20% formamide, 10
mM Tris-HCl pH 7.4. The R-loop molecules were picked up on parlo-
dion coated grids, stained with uranyl acetate and shadowed with
platinum-palladium[5,20]. Electron micrographs were taken with a
Siemens Elmiskop 101 at 60 kV. The length of the molecules was
measured with a Hewlett Packard 9864B digitizer connected with
a 9810 calculator.

As the length of both the proximal and distal spacers of the
rDNA gene are close to 2 kb, it was not possible to orientate
the snapback R-loop molecule containing non-spliced pre-35S rRNA.
Therefore, a restriction had to be put on all molecules that were
included in the statistics: the R-loop must include the region
containing the intervening sequence of the gene independent of
how the molecule is orientated. Of the analysed molecules, 95%
fulfil the requirement that the loop proceeds to at least 2.9 kb
from the ends of the molecule. The remaining 5% could be ascrib-
ed either to non-terminated rRNA chains or to break down pro-
ducts. The size distribution of the included molecules fit to a
normal distribution.

RESULTS

The rDNA exists in the macronucleus as extrachromosomal
molecules with a palindromic structure[2,3]. Figure 1 represents a
map of rDNA from Tetrahymena thermophila strain 1868-7 based on
the data obtained by Engberg et al.[9]. The restriction endonucle-
ase cleavage sites of HhaI and BglII are indicated on the map.
Incubation of nucleoli isolated from Tetrahymena thermophila
with ^{32}P-labelled nucleoside triphosphate gives in the presence
of Mg^{2+}, Mn^{2+} or Co^{2+} faithful transcription of the rRNA gene by
the endogenous RNA polymerases. Studies of the synthesized
transcripts on 1.8% gels demonstrate products with sizes around
6 kb (Figure 2A). In addition to the larger products a small
fairly diffuse band with a size around 0.4 kb appears in the
samples incubated in the presence of Mg^{2+}. However, if the same
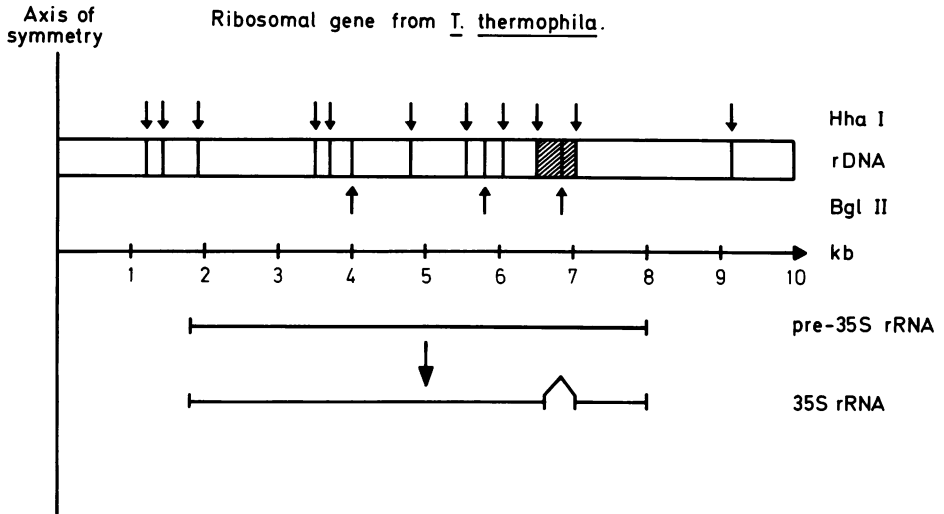
Figure 1. Map of rRNA gene from <u>Tetrahymena thermophila</u>, based on Engberg et al.[9]. The cleavage sites for the restriction endo-nucleases HhaI and BglII are indicated by arrows. The restriction fragments containing the intervening sequence are shaded.

transcripts are analysed on a 3.0% gel a well defined band with a size of 0.4 kb is found in the samples (Figure 2B). The band is not present in the samples incubated in the presence of Mn^{2+} or Co^{2+} (Figure 2B). Detailed size studies on various types of gels determined the size of RNA in the band to 420±20 bases, identical to the size of the intervening sequence in the rDNA from <u>Tetrahymena thermophila</u>[4-9]. In order to identify and characterize the 0.4 kb RNA further, hybridization studies were performed to restriction fragments of rDNA by the method of Southern[18]. Figure 3A shows an ethidium bromide stained gel after double digestion of rDNA with the restriction enzymes HhaI and BglII. Two fragments with a size of 0.30 and 0.20 kb, respective-ly, cover the entire region of the intervening sequence (see the restriction map in Figure 1). After transfer of the DNA fragments to nitrocellulose filters, hybridization to the total transcript and to the 0.4 kb band, both labelled with ^{32}P, was performed. The corresponding autoradiograms demonstrate that the total transcripts hybridize to most of the bands (Figure 3B), while
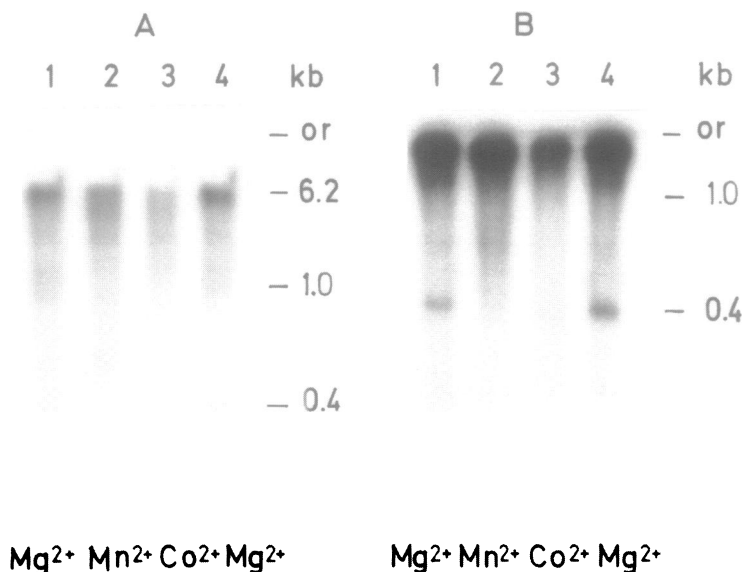
Figure 2. Size analysis of in vitro synthesized rRNA from isolated nucleoli analyzed on denaturing 1.8% (A) and 3.0% (B) agarose gels. Nucleoli were incubated for 30 minutes with reaction mixture at 25°C in the presence of 5 mM MgCl$_2$ (Lanes 1 and 4), 0.25 mM MnCl$_2$ (Lane 2), 0.15 mM CoCl$_2$ (Lane 3). After electrophoresis the gels were exposed to autoradiography.

the 0.4 kb RNA hybridizes exclusively to the 0.30 and 0.20 kb rDNA fragments (Figure 3C). Corresponding results have been obtained by hybridization of the 0.4 kb RNA to BamHI   HindIII, HhaI or BglII restriction fragments of rDNA: the 0.4 kb RNA hybridizes specifically to those fragments containing the intervening sequence. Quantitative studies demonstrate that the 0.4 kb RNA accounts for about 6% the total RNA synthesized. Based on the knowledge that reinitiation does not occur in vitro and on the assumption that the RNA polymerases are randomly distributed over the transcription unit, it can be calculated that the intervening sequence constitutes about 11% of the in vitro synthesized. This suggests that the 0.4 kb RNA has arisen from nearly 50% of the synthesized 6 kb transcripts. Thus, the first part of our results demonstrate that an 0.4 kb RNA with a sequence homologous to the intervening sequence in the rDNA is excised and that the
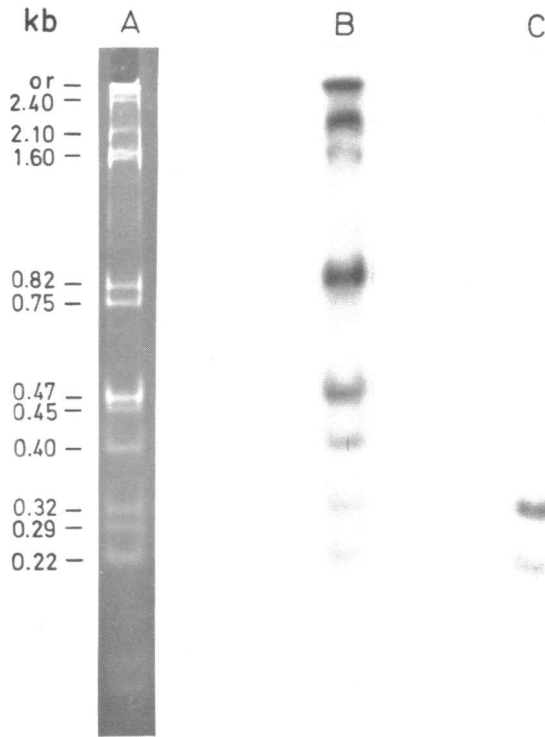
Figure 3. (A) Ethidium bromide stained fragments of rDNA after digestion with restriction endonuclease HhaI plus BglII and separation on a 4% agarose gel. (B) Autoradiogram of Southern blot of restriction endonuclease fragments from A hybridized to total ^{32}P-labelled rRNA. (C) Autoradiogram of Southern blot hybridized to the ^{32}P-labelled 0.4 kb RNA fragment.

enzyme activity involved in the endonucleolytic cleavage around the intervening sequence is associated with the purified nucleolar structure.

It is possible to separate the process of transcription from the endonucleolytic cleavage at the intervening sequence as seen in Figure 4. Samples of isolated nucleoli were incubated for 30 minutes in the presence of Mg^{2+} or Mn^{2+} in order to get full-size products (Lanes 1 and 2). In both cases the reaction are fully completed within 30 minutes. Excision of the 0.4 kb RNA does only occur in the presence of Mg^{2+}. However, when a
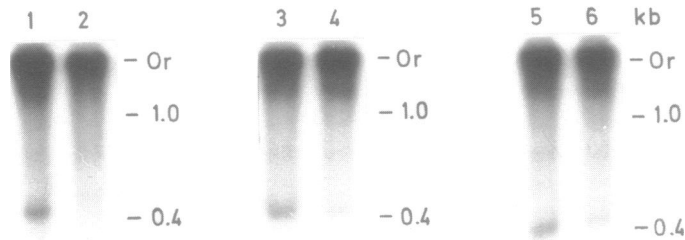
Figure 4. Processing of in vitro synthesized pre-35S rRNA. Isolated nucleoli were incubated for 30 minutes at 25°C in an α-^{32}P-UTP containing reaction mixture with 5 mM Mg^{2+} (Lanes 1, 3, and 5) or 0.25 mM Mn^{2+} (Lanes 2, 4, and 6). The samples were thereafter incubated with a chase of 1.0 mM cold UTP and 5 mM Mg^{2+} for 0 minute (Lanes 1 and 2), 5 minutes (Lanes 3 and 4), and 12 minutes (Lanes 5 and 6), respectively. The samples were analyzed on 3% denaturing gels and exposed to autoradiography.

sample, incubated with Mn^{2+}, is reincubated in the presence of Mg^{2+} the 0.4 kb RNA band appears demonstrating that excision of the intervening sequence is a posttranscriptional event. The excision levels off after 5 minutes of incubation, as it is seen from Figure 4, where samples have been reincubated with Mg^{2+} for 5 minutes (Lanes 3 and 4) and 12 minutes (Lanes 5 and 6).

As the endonucleolytic cleavage can be separated from the transcriptional event itself it is possible to investigate the excision in more details. We have studied the effect of proflavine on this process. Proflavine is an intercalating agent[21,22,23] which binds to nucleic acids in their double standed regions and it has previously been reported to prevent processing of RNA in vivo[24,25] and in isolated nuclei[26]. Furthermore, we have reported that proflavine inhibits correct transcription termination in isolated nucleoli[27]. The effect of proflavine on the excision of the transcribed intervening sequence can be studied by addition of the drug to nucleoli containing pre-35S rRNA synthesized in

the presence of Mn^{2+}. Figure 5 demonstrates the effect of in-
creasing concentrations of proflavine on the processing of pre-
synthesized precursor rRNA. Excision of the 0.4 kb RNA occurs at
concentrations up to 20 μg/ml of proflavine. At higher concentra-
tions, the reaction is fully inhibited, suggesting that double
stranded RNA structures are involved in the recognition and/or
the excision process. The given concentrations of proflavine do
not have any effect on the elongation process[27].

In the studies of the splicing activities of yeast pre-tRNA,
it has been possible to separate the endonucleolytic activity
from the ligation step (see ref. 28 for a review). This suggests
that endonucleolytic cleavage of the pre-rRNA is not necessarily
linked to the ligation under the given conditions. If only the
excision takes place in isolated nucleoli, two larger RNA frag-
ments would accumulate in addition to the 0.4 kb transcribed in-
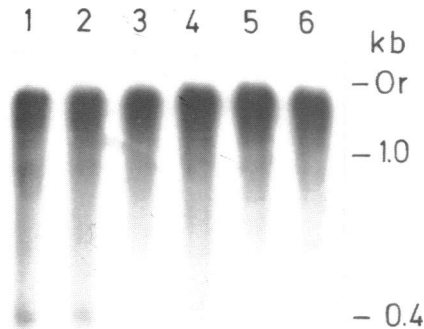
Figure 5. Effect of proflavine on processing of pre-35S rRNA.
Isolated nucleoli were incubated for 30 minutes in a ^{32}P-UTP con-
taining reaction mixture in the presence of 0.25 mM Mn^{2+}. There-
after the samples were added 5 mM Mg^{2+}, 1 mM cold UTP, and pro-
flavine in concentrations from 0-50 μg/ml: (1) 0 μg/ml, (2) 10
μg/ml, (3) 20 μg/ml, (4) 30 μg/ml, (5) 40 μg/ml, and (6) 50 μg/ml.
After 20 minutes of incubation at 25°C the samples were analyzed
on 3% denaturing agarose gels and exposed to autoradiography.

tervening sequence. The two RNA fragments would represent the 5'
proximal fragment with a size of 4.7 kb and the 3' terminal frag-
ment with a size of 0.9 kb, as transcription in the performed ex-
periments is terminated correctly. Technically it is difficult to
distinguish the 4.7 kb fragment from the other high molecular
weight molecules on the 3.0% gel, but the 0.9 kb band should ea-
sily be identified. In addition, the 0.9 kb band would contain
more than twice the radioactivity of the 0.4 kb RNA. Normally we
do not find a band in the 0.9 kb region, but in a few prepara-
tions a faint band is observed. The band accounts in these expe-
riments for less than 1% of the total transcript. This might in-
dicate that excision and ligation are normally linked processes
in the isolated nucleoli.

In order to obtain a final proof of RNA ligation in isolat-
ed nucleoli, we performed R-loop studies on the total RNA. Bio-
chemical studies have demonstrated that 95% of the RNA content
in isolated nucleoli are in vivo terminated chains. This high
percentage indicates that processing of the intervening sequence
or alternatively transport of the spliced transcripts from the
nucleoli is rate limiting. Consistent with the biochemically
determined pool size, we find by electron microscopic studies
that most of the RNA molecules (95%, cf. Materials and Methods)
found in RNA-DNA hybrids are close to full size. Thus, the average
length of the non-processed molecules was 6175 ± 270 bp (n = 114).
These non-processed pre-35S rRNA molecules account for 79% of the
registered molecules, while the remaining 21% are spliced 35S
rRNA molecules (cf. Table 1). The presence of a high amount of
unspliced molecules in the isolated nucleoli is consistent with
the in vivo studies by Din et al.[5] showing a half life time of
pre-35S rRNA of approximately ½ min for T. thermophila.

R-loop analyses of the total RNA from nucleoli incubated
under conditions similar to those under which the biochemical
studies were performed gave the results listed in Table 1. When
the incubation was carried out in the presence of Mg^{2+}, the per-
centage of spliced 35S rRNA molecules is increasing from 21% to
51%. In contrast, in the presence of Mn^{2+} or Mg^{2+} plus 40 µg pro-
flavine/ml the percentages remain low, 18 and 22%, respectively.
This demonstrates that isolated nucleoli are able to ligate RNA-
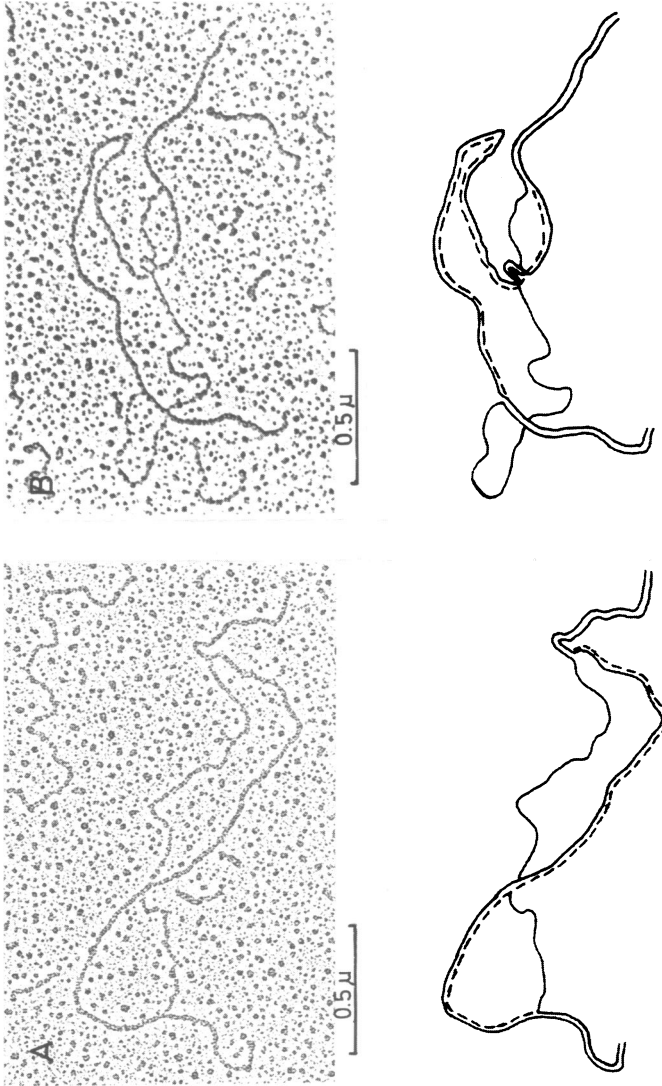
Figure 6. Electron micrographs of R-loop molecules. Isolated nucleoli were deproteinized, and nucleic acids precipitated and hybridized under conditions, where snap-back molecules of rDNA are formed. The preparations were spread for electron microscopy as described under Methods. R-loop formed with (A) pre-35S and with (B) 35S rRNA molecules.

Table 1. Summary of the electron microscopic studies of processing of rRNA in isolated nucleoli.

Incubation Conditions	total number analyzed (n)	pre-35S rRNA		35S rRNA	
		percent	(n)	percent	(n)
non-incubated	144	79%	(114)	21%	(30)
incubated in the presence of XTP and Mg^{2+}	113	49%	(55)	51%	(58)
incubated in the presence of XTP and Mn^{2+}	111	82%	(91)	18%	(20)
incubated in the presence of XTP, Mg^{2+}, and proflavine (40 μg/ml)	113	78%	(88)	22%	(25)

RNA under the conditions, where the endonucleolytic cleavage of pre-35S rRNA takes place.

On the basis of R-loop analyses we have constructed a map of the rDNA of T. thermophila as shown in Figure 7. This map fits closely with the recent S_1-mapping data by Engberg et al.[9].

DISCUSSION

We have demonstrated that the enzyme activities involved in splicing of the rRNA precursor can be isolated together with transcriptionally active nucleoli and therefore seem to be strongly associated with the nucleolar structure. The evidence for endonucleolytic cleavage around the splice junction comes

| $\bar{X}$ | 1840 | 4780 | 400 1020 | 1960 | n = 133 |
| σ_n | 190 | 210 | 50 110 | 200 | |

axis of
symmetry
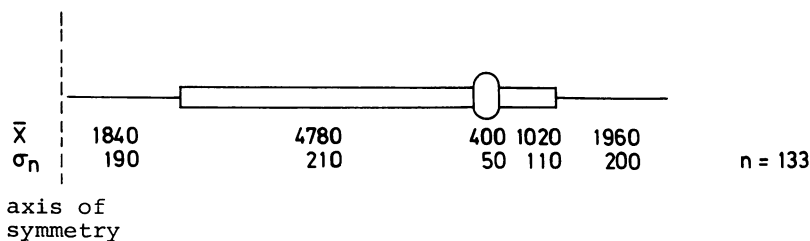
Figure 7. Map of rDNA from Tetrahymena thermophila based on R-loop studies.

from biochemical studies and the evidence for ligation mainly
from electron microscopic analyses. Previously, it has been de-
monstrated that endonucleolytic cleavage or processing of diffe-
rent precursor rRNA molecules can take place in isolated nu-
clei[8,29-31]. Our results are the first evidence that the splicing
activities can be purified associated with a specific gene in its
chromatin form. The localization of the processing enzymes on the
nucleolar chromatin is a contrast to the yeast tRNA system de-
scribed by Abelson and coworkers, where the splicing activities
are purified from a ribosome pellet[32-34]. It is not possible,
based on the present data, to estimate how many fold the enzymes
involved in the splicing of the pre-rRNA from Tetrahymena have
been purified with respect to protein. However, in the isolated
nucleoli more than 90% of the DNA is rDNA and the nucleolar
chromatin is purified more than 200 fold with respect to protein.

The splicing of the pre-rRNA occurs posttranscriptionally
in vitro, as it is seen from the experiment with Mn^{2+} and Co^{2+}.
In these experiments the endonucleolytic cleavage at the splice
junction can be separated from the transcriptional event. The re-
sults are consistent with the data from in vivo experiments by
Din et al.[5]. Also the electron microscopic data showing the accu-
mulation of large amounts of non-processed rRNA precursor agree
with the in vivo data[5].

The observation that the endonuclease activity is absent in
the presence of Mn^{2+} or Co^{2+}, while transcription takes place
with nearly normal rate, provides us with an useful tool for ac-
cumulation of unspliced precursor molecules. It is not yet known
if the method can be applied to other splicing systems, but it
might be a powerful method for accumulation of substrates for
various processing enzymes.

The sequence around the splice junction of the rRNA gene
from Tetrahymena differs from the sequences of the splice junc-
tion of both RNA polymerase II and III genes[7,28,35]. Also the
processing enzymes seem to differ in some of their requirements.
Thus, the yeast tRNA system required high concentrations of ATP
(>1 mM) in order to ligate the two fragments[33,34,36], while we
do not find this requirement for high ATP in the Tetrahymena
system. A possible explanation might be that the enzymes in the

nucleolar system still are in the right structural arrangement,
while the properties of the yeast enzymes might have changed
during the solubilization from a functional structure.

Comparison of the data on in vitro processing of in vivo
and in vitro synthesized transcript, obtained from R-loop (Table
1) and gel studies (Figure 4), respectively, shows that a maximum
of about 50% excision-ligation is obtained in vitro in both
cases. This indicates that the processing enzymes do not distin-
guish between in vitro and in vivo synthesized pre-rRNA.

The inhibitory effect of proflavine on the splicing event
seems to be a general phenomenon for many processing systems[24-26]
suggesting that RNA-RNA interactions are involved in the process.
We have demonstrated that proflavine also prevents transcription
termination on the rRNA gene[27]. Therefore, all results on profla-
vine described in this paper are performed on correctly terminat-
ed in vitro transcript synthesized in the presence of Mn^{2+}. How-
ever, it is not in the in vivo experiments reported by others
possible to distinguish between the effect of proflavine on the
process of termination and on the processing event, itself. Re-
cently, experiments done in this laboratory (manuscript in pre-
paration) on termination-deficient chromatin have demonstrated
that correct termination is an absolute requirement for splicing.

The great potential of the experimental system described for
studies of the splicing of the ribosomal RNA precursor is based
on the fact that splicing can take place under defined condition,
where several events can be controlled, separately. Experiments
on the dissociation of the splicing activities from the nucleolar
structure are now in progress.

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