Chapter 6

In vitro synthesis and processing of ribosomal RNA
6.1. Introduction

Ribosomal RNA (rRNA) is a major component of ribosomes, the universal cellular machines for protein synthesis. It also accounts for nearly 80% of total RNA in the cell. This rRNA plays defined roles in protein synthesis, which is conserved throughout evolution such as binding of messenger RNAs (mRNAs), transfer RNAs (tRNAs) and most probably catalysis of peptidyl transfer. In prokaryotes this rRNA is synthesized by RNA polymerase as a long precursor molecule called pre-rRNA consisting of the large subunit ribosomal RNA (LS rRNA) and small subunit ribosomal RNA (SSrRNA) along with the 5S rRNA. In eukaryotes the situation is different. Here the pre-rRNA is synthesized by RNA polymerase I and the pre-rRNA contains 5.8S rRNA in place of 5S rRNA while the 5S rRNA is synthesized independently. The pre-rRNA molecule is then acted upon by a series of endonucleolytic cleavage steps generating the mature rRNA molecules. In the last few years a considerable effort has been given to the understanding of maturation of rRNAs from the pre-rRNA molecule and it is found that the maturation steps are distinct for each of the representative kingdom, namely eubacteria, archaeabacteria and eukaryotes.

In simple prokaryotes, including eubacteria and archaeabacteria, the 16S and 23S rRNAs are cotranscribed with 5S rRNA and one or more intergenic and distal tRNAs (Srivastava and Schlessinger, 1990; Kjems and Garret, 1990; Dennis, 1991; Garret et al., 1991). In eubacteria, a universal feature of the transcription unit is the presence of large inverted sequences that surrounds the 16S and 23S rRNA genes. These repeats are predicted to form helical structures in the nascent transcript causing the rRNA molecule to be looped out. It also contains the recognition features for endonuclease activities that excise precursor 16S and precursor 23S rRNA from the primary transcript. In E.coli and related eubacteria the enzyme RNase III is responsible for these initial excisions. The enzyme recognizes duplex RNA of generally undefined sequences, often with one or more bulge or unpaired nucleotides near the site of cleavage. Cleavage occurs at staggered positions on each of the two strands and produces a 5' phosphate and 3' hydroxyl products (Court, 1993; Kharrat et al., 1995). Subsequent trimming at the 5' and 3' ends is required to produce the mature 16S and 23S rRNA (Apirion and Miczac, 1993).

Though the rRNA operons in archaeabacteria are similar to those of eubacteria, the enzymology of precursor processing is somewhat different (Dennis, 1991; Garret, 1991). Unlike eubacteria, the predicted processing helices of archaeabacteria contain a well-defined structural motif that serves as the recognition site for the excision endonuclease. The motif consists of two
three-base bulges on opposite strands, which are separated by precisely four helical base pairs near the center of a much larger helical structure. Cleavage within the bulge results in products with a 5' hydroxyl group and 3' phosphate (Thompson et al., 1989). This excision endonuclease is a tRNA intron endoribonuclease, an RNase III-like enzyme (Thompson and Daniels, 1988). But in *Sulfobus acidocaldarius*, an archaeabacterium, two distinct biochemical mechanisms are used to initiate the excision of precursor 16S and 23S rRNAs from the primary transcript. Here, at least three separate precursor endonucleolytic cleavages occur within the 144-nucleotide-long 5' ETS region of the rRNA operon primary transcript. These are at position -99, -31 and +1. The processing activity contains five or six polypeptide components and perhaps as many as ten different small RNA components. One of the RNA components exhibits sequence and structural similarities to eukaryotic U3 snoRNA. RNase H digestion and micrococcal nuclease sensitivity suggest that the U3-like RNA is an essential component of the pre-rRNA processing RNP endonuclease (Potter et al., 1995). Cleavages at the -99 and -31 sites depend on the presence of a purine at 5' end and a uracil two nucleotide 3' to the scissile phosphodiester bond (Russell et al., 1999).

In the larger and more complex eukaryotic cell, transcription and processing of rRNA and assembly into ribosomal subunits occur in the nucleolus (Melesa and Xue, 1995). The pre-rRNA is transcribed by RNA Polymerase I and contains a 5' external transcribed spacer (ETS), the 18S, the first internal transcribed spacer (ITS1), the 5.8S rRNA, the second internal transcribed spacer (ITS2) and the 28S rRNA. As proteins begins to associate with the RNA, the precursor is cleaved and the excised 20S rRNA is rapidly converted to its mature 18S form. Processing and maturation of the 5.8S and the 28S rRNA occurs more slowly (Gerbl et al., 1990; Sollner-Webb et al., 1994). In yeast *Saccharomyces cerevisiae*, the most well studied system, the 35S pre-rRNA is cleaved at at least 8 different positions (Fig. 6.1). Depletion studies of Rok1p, a putative RNA helicase, inhibits pre-rRNA processing at sites A0, A1 and A2, thereby blocking 18S rRNA synthesis (Venema et al., 1997). The site at A3 in ITS1 is cleaved by RNase MRP in association with a putative RNA helicase Dbp3p (Schmitt and Clayton, 1993; Weaver et al., 1997). Processing at all these four sites are inhibited by genetic depletion of a nucleolar protein Rrp5p providing evidence of close interconnections between these processing events (Venema and Tollervey, 1996). Thereafter the 5' end of the 5.8S rRNA is generated by exonuclease activity (Henry et al., 1994). Contemporaneously with the processing reaction at the 5' end of 5.8S, the B2 site in the 3' external transcribed spacer (ETS) is cleaved. The mechanism and precise relative timing of this cleavage is not known. The 27SB pre-rRNAs (Fig. 6.1) are processed to mature 5.8S and to 25S rRNA by removal of the ITS2 in a set of reaction not known to require snoRNPs. The 3' end of
mature 5.8S is generated by exonuclease activity (Mitchell et al., 1996). Different small nucleolar RNAs (snoRNAs) including U3, U8, U13, U14, and U15, have been identified in ribonucleoprotein complexes and implicated in rRNA processing (Beltram and Tollervey, 1995; Cavaille et al., 1996; Hughes, 1996; Liang et al., 1997; Peculis, 1997). The most abundant snoRNA, U3 has been shown to be required for an early cleavage within the external transcribed spacer and has been implicated along with U14 in subsequent steps in 18S rRNA maturation (Savino and Gerbi 1990; Kass et al 1990; Li, et al 1990; Hughes and Ares 1991; Sharma and Tollervey, 1999). In yeast RNase III is involved in processing of the pre-rRNA but do not pair the ends of the pre-rRNAs to generate RNase III substrate (Elela et al., 1996). Instead it relies on more locally restricted interactions.

Thus in the three different kingdoms an overlapping set of components has been used to develop different strategies for pre ribosomal RNA processing (Fig. 6. 2).

Though much is known about the biogenesis of rRNAs in prokaryotes and eukaryotes, very little knowledge is available till date for the early diverging eukaryotes, the protozoans in general and Giardia lamblia in particular. Much work has been done on the ciliated protozoa Tetrahymena. In Tetrahymena the rDNA is present extrachromosomally (Gall, 1974; Engberg, 1974) and each rDNA molecule has a palindromic sequence symmetry and is transcribed bidirectionally (Karrer, 1976; Engberg et al., 1976) to give a 35S RNA as the primary transcript (Niles, 1978; Sutton et al., 1970). This primary transcript contains an intervening sequence (IVS) located in the 26S rRNA coding region (Wild and Gall, 1979) and excision of the IVS is the first discernible step in the processing of this transcript (Cech and Rio, 1979). Once the IVS is cleaved then the endonucleolytic steps occur in the usual 5' to 3' direction resulting in the production of mature 17S, 5.8S and 26S rRNA (Wellauer and David, 1974; Reeder et al., 1976). In the malarial parasite Plasmodium the gross architecture of the rRNA genes are similar to that of eukaryotes except that the eukaryotes the rRNA genes of Plasmodium are present only in few copies (Gunderson et al., 1987; Vaidya et al., 1989; Yap et al., 1997; Thompson et al., 1999) and are dispersed on different chromosomes (McCutchan et al., 1995). Surprisingly in addition to this nuclear rRNA genes the parasite also contains rRNA genes on extrachromosomal DNA elements (Vaidya et al., 1989; Yap et al., 1997). Also the sequences of the nuclear rRNA genes of Plasmodium are different and expression of these genes are stage specifically regulated, resulting in different ribosome types in specific parts of their life cycle (Gunderson et al., 1987; Thompson et al., 1999).

Very little is known about the biogenesis of rRNA of Giardia. In Giardia the rRNA genes are chromosomal and located in the telomeres (Adam, R. D. 1991). The 5,566bp rDNA
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Fig. 6. 1. Processing of pre ribosomal RNA transcripts in *S. cerevisiae* (Morrisey and Tollervey, 1995).

Fig. 6. 2. Processing of pre ribosomal RNA in representatives of different kingdoms. Processing sites are indicated by arrows. The position of the mature rRNA sequences are indicated by dark boxes and circles (Elela et al., 1996).
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gene (Healey, et al., 1990) encodes the large subunit (28S), small subunit (16S), and 5.8S forms of sizes 2400, 1400 and 138 nucleotides respectively, each of which is smaller than its counterparts in other eukaryotic organisms. Sequence analysis of the gene encoding the small subunit rRNA shows greater similarity to the archaebacteria sequence than do the sequences from other eukaryotes. Computer aided analysis for potential secondary structure indicated that ITS1 and ITS2 display no significant complementarity to each other or to the processed rRNA regions 5' to SS rRNA or 3' to LS rRNA (Edlind et al., 1990). This rules out the formation of long-range base pairing and RNAase III-type processing as seen in bacterial pre-rRNA. In contrast the sequence analysis suggested that the sequence GCGCCCC, in a hairpin configuration might function as the processing signal (Edlind et al., 1990). So rRNA of *Giardia lamblia* has the potential to provide a useful model for studies of rRNA structure, function and processing.

As has been mentioned above the pre-rRNA in eukaryotes are synthesized and processed in the nucleolus. The processing of rRNAs can be studied both *in vivo* as in *Xenopus* (Savino and Gerbi, 1990) and yeast (Hughes and Ares, 1991) or *in vitro* as in *Tetrahymena* (Zaug and Cech, 1980). Lack of any defined gene transfer system for *Giardia* has led us to study the processing *in vitro*. A number of powerful methods like run-on transcription, primer extension and RNase protection analysis are available for studying the rRNA processing *in vitro*. In this chapter attempts have been made to understand some of the biochemical pathways of biogenesis of giardial rRNAs taking help of the above mentioned tools. The 5' ETS which lies within the IGS of the pre-rRNA has been chosen for analysis, since this is the region from which the processing of the pre-rRNA starts.

6.2. Methods

6.2.1. Preparation of active nuclei

Active nuclei of *Giardia lamblia* were prepared following the method of Marzluff and Murphy, with little modification (Marzluff et al., 1973). Approximately $2 \times 10^8$ cells were washed in 1X PBS and pelleted for 10 min at 1600rpm. The cells were suspended in 2 volumes of swelling buffer (section 3.4.5.1) and incubated on ice in a dounce homogenizer for 15 min to swell the cells. The cells were then lysed by homogenization using tight fitting pestle. Cell lysis was monitored by phase contrast microscopy. The lysate was then centrifuged at 1600rpm and the supernatant was discarded. The nuclei were suspended in storage buffer (section 3.4.5.2) at a concentration of approximately $2 \times 10^8$ nuclei/ml and stored at -70°C.
6.2.2. Isolation of total RNA

Total RNA from *G. lamblia* was isolated as mentioned in section 4.5.

6.2.3. Nucleic Acid hybridization

6.2.3.1. Northern Hybridisation

RNA was separated in 1% MOPS formaldehyde agarose gel. After the run the gel was washed twice in sterile water for 15min each. The RNA bands separated in gel was then subjected to transfer onto Zeta probe membrane (Bio-Rad) by capillary action overnight in presence of 20X SSC (section 3.4.4.1). After the transfer the membrane was washed with 5X SSC, air-dried and the RNA was crosslinked under UV. The membrane was prehybridized for 2hrs at 42°C with prehybridization solution containing 5X Denhardt's (section 3.4.4.2), 5X SSC, 0.1 SDS and 50% formamide in a hybridization oven. Denatured radiolabeled probe was added to this and hybridization was carried out overnight at 42°C. Subsequently the membrane was rinsed once with 5X SSC, 0.5% SDS at 65°C for 5 min, twice with 0.1X SSC, 1% SDS at 50°C for 40 min and once with 2X SSC for 5min at room temperature. The membrane was dried and exposed to X-ray film.

6.2.3.2. Slot blot hybridization

Slot blot hybridization was performed on a nylon membrane using Bio-Dot SF apparatus (BioRad) according to the instruction given by the manufacturer. Nascent RNA synthesized by run on transcription was loaded in each slot. Prior to blotting NaOH and EDTA was added to the RNA sample, to a final concentration of 0.4 M NaOH and 10 mM EDTA. Samples were heated to 100°C for 10min to ensure complete denaturation. The RNA solution was then neutralized by adding equal volume of cold 2M ammonium acetate, pH 7.0. After blotting the membrane was washed with 6X SSC, dried and the RNA crosslinked under UV light. The membrane was hybridized with labeled DNA probe according to the protocol given in northern hybridization.

6.2.4. Primer extension analysis

Primer extension was done to map the 5' termini of pre-rRNA and to detect precursors and processing intermediates of pre-rRNA. Total RNA was hybridized with an excess of single-stranded DNA primers radiolabeled at their 5' terminus. Reverse transcriptase was then used to
extend these primers to produce cDNA complementary to the RNA template. The length of the resulting end-labeled cDNA was measured by electrophoresis through a polyacrylamide gel under denaturing conditions, which reflected the distance between the end-labeled nucleotide of the primer and the 5' terminus of the pre-rRNA. We have used the primer extension analysis to study the transcription start site and the processing intermediates of pre-rRNA of *Giardia lamblia*.

Total RNA (50μg) in 300 μl volume was mixed with 20μl of end-labeled primer (10^6 cpm. cerenkov radioactivity) and 0.1 volume 3 M sodium acetate. 2.5 volume of 100% ethanol was added to it and the RNA was precipitated by centrifugation at 12,000g. The pellet was washed with 70% alcohol and air dried. 8μl TE buffer (section 3. 4. 7. 1) and 2μl 5X annealing buffer (section 3. 4. 7. 2) was added to the pellet. The sample was incubated at 75°C for 1.5 min and quickly transferred to 58°C water bath. The sample was incubated for 1 hr to hybridize the primer to the RNA. The mixture was allowed to cool to room temperature and 40μl of RT mix (3. 4. 7. 3) was added and mixed by brief vortexing. It was incubated at 37°C for 1 hr. Reaction was terminated by 0.1 volume of 3M sodium acetate. The mixture was treated with phenol:chloroform:isoamyl alcohol (25:24: 1) and precipitated with 100% ethanol. The pellet was dried in vacuum concentrator. It was suspended in 1X RNA loading dye (3. 4. 3. 1. 3) and ran in 5% denaturing acrylamide gel.

### Table 6.1. Primers used in primer extension analysis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>position</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>17mer</td>
<td>5’- GCC GTC CGC CGC GTC CC-3’</td>
<td>4994-5011</td>
<td>69°C</td>
</tr>
<tr>
<td>AS2</td>
<td>5’- AGG ACT GCA TAT CAC GGC-3’</td>
<td>4539-4555</td>
<td>61°C</td>
</tr>
</tbody>
</table>

The 17mer primer was designed corresponding to the complementary sequence within SS rRNA. AS2 primer corresponds to the complementary sequence within ETS.

### 6. 2. 5. Nuclease protection assay

P³² labelled RNA probes in 30μl hybridization buffer (section 3. 4. 8. 1) was used to suspend 50μg of pelleted total RNA and the mixture was incubated at 85°C for 10 min to denature the RNA. The mixture was quickly transferred to a 55°C water bath and incubated overnight. 350μl of ribonuclease digestion buffer (3. 4. 8. 2) was added to the mixture and incubated at 30°C for 60 min. Then 10μl of 20% SDS and 2.5μl of 20mg/ml proteinase K was added and incubated at 37°C for 15 min. The mixture was extracted with 400μl phenol:
chloroform: isoamylalcohol (25: 24: 1) and then ethanol precipitated in presence of glycogen (20mg/ml). The pellet was redissolved in RNA loading dye, denatured at 85°C for 3 min, and analyzed on 5% denaturing polyacrylamide/urea gel at 300volts. The gel was dried and autoradiographed.

6.3. Results

6.3.1. Standardisation of Run on transcription reaction

Run-on transcription reaction is a good in vitro method for studying transcription. In this method isolated nuclei containing preformed transcription complexes are allowed to elongate in the presence of a labeled NTP. The radiolabeled, nascent RNAs that are formed mimic the RNA that is present in vivo in the nucleus.

6.3.1.1. Selection of assay buffer for Run on transcription reaction

Reaction was done in a total volume of 25μl containing different combinations of concentrations of Hepes-KOH (pH 7.9), MgCl₂, NaCl, and 10mM creatine phosphate, 4mM ATP, 1mM each of GTP and CTP, 10μM UTP, and 25μCi of α³²UTP, 2mM DTT, 12.5% glycerol and 1u/μl RNasin and 10⁹ nuclei/ml (section 6.2.1). The reaction was incubated at 25°C for 30 min. Incorporation into RNA was quantitated by DE81 filter assay. Faithful incorporation was obtained in presence of 15mM Hepes-KOH (pH 7.9), 5mM MgCl₂ and 50mM NaCl.

6.3.1.2. Kinetics of Run on transcription reaction

Transcription reaction was done as above in 25μl volume in presence of α³²UTP. 1μl of reaction mixture was taken at different time intervals and spotted onto DE81 filter paper and amount of radioactive incorporation was measured as described by Sambrook et. al. (Sambrook et al., 1991). The incorporation was plotted against time (Fig. 6.3A). It was found that the maximum incorporation was at 15 minutes after which there was a rapid fall in incorporation. Hence the optimum time period of transcription reaction was taken as 15 minutes.

The product formed was treated with proteinase K and DNase I at 37°C, then extracted with phenol: chloroform: isoamylalcohol (25: 24: 1) and precipitated with ethanol in presence of 0.3 (M) sodium acetate. The product was analyzed on 1% MOPS formaldehyde agarose
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denaturing gel (Fig. 6. 3B). Distinct bands against a smeary background was obtained starting from around 3Kb. This was expected as run on transcription generated all the different type of RNAs viz rRNA, mRNAs and tRNAs. But no full-length pre-rRNA (expected size of over 3Kb) was seen. This may be due to the fact that processing of the pre-rRNA after transcription takes place immediately.

6.3.1.3. α-amanitin sensitivity of Run on transcription reaction

α-amanitin, a poison isolated from *Amanita phalloides*, can be used to distinguish the three RNA polymerases present in eukaryotes. RNA polymerase I that transcribes ribosomal RNAs is insensitive to α-amanitin while RNA Polymerase III which transcribes the 5S and different tRNAs are moderately sensitive. In contrast the RNA polymerase II which transcribes the protein coding genes, is highly sensitive to the poison and can be inhibited by as little as 100μg/ml of the drug (Alberts et al., 1994). To analyze the mode of transcription in *G. lamblia*, which is known to possess three types of RNA polymerases (Lanzendofer et al., 1992) the above reaction was carried out in absence and presence of α-amanitin at a concentration as high as 200μg/ml. Incorporation was monitored by filter binding assay. It was found that the transcription machinery was sensitive to α-amanitin and the incorporated radioactivity was 2 fold lower in presence of α-amanitin (Fig. 6. 4. A).

6.3.1.4. Determination of the type of RNA transcribed in presence of α-amanitin:

To assess which of the three RNA polymerases was sensitive to the α-amanitin, cold run on transcription was carried out as above in presence and absence of α-amanitin (200μg/ml) in 100μl volume. Control reaction, containing no rNTPs was done side by side so that no new RNA was synthesized. After 15 min the reaction was terminated by adding 1%SDS and 100μg/ml Proteinase K, followed by incubation at 25°C for 15 min. RNA was extracted with phenol: chloroform: isoamylalcohol and ethanol precipitated in presence of 3M sodium acetate. Contaminating DNA was removed by treatment with RNase free DNase I (Bohringer Mannheim) and again extracted with phenol: chloroform: isoamylalcohol. The RNA was obtained by ethanol precipitation in presence of 2.5M ammonium acetate. The products were then slot blotted onto zeta probe membrane. The membrane was air dried and UV crosslinked for 2 min. The membrane
Fig. 6. 3. A. Profile of run-on transcription using *Giardia* nuclei. Samples (1μl) were taken from run-on transcription reaction at 0, 2, 5, 10, 15 and 30 min after the addition of α P32 UTP to the transcription buffer. The incorporation in cpm is plotted against the time.

Fig. 6. 3. B. The run-on transcripts analyzed on 1% MOPS formaldehyde agarose gel. The positions of prokaryotic LS (23S) and SS (16S) rRNA are shown by arrowhead.
Fig. 6.4. A. α-Amanitin sensitivity of run on transcription. Samples (1μl) were taken at 0, 2, 5, 10, 15 and 30 min from run-on transcription reactions carried out in absence (series 1) and presence (series2) of α-amanitin after the addition of the radionucleotide to the transcription buffer. The radioactivity was measured as described by Sambrook et. al.

Fig. 6.4. B. Slots blot hybridization of run-on transcription of rRNA genes using Giardia nuclei. Cold nuclear RNA was synthesized in presence (lane 1) and absence (lane 3) of α-amanitin and slotted in wells. Lane 2 served as control where run on transcription reaction was done without any cold NTPs from outside. The membrane was hybridized to P32-labeled rDNA probe.
was hybridized with the radiolabeled rDNA probe 1 (Fig. 6. 5) representing a part of SSrRNA and a part of 5'ETS.

It was found that the nascent RNA synthesized both in presence and absence of α-amanitin hybridized to the rDNA probe with similar intensities (Fig. 6. 4B) in lane 1 and 3 whereas lane 2 demonstrates the endogenous rRNA synthesis. This indicates that transcription of the rRNA is insensitive to α-amanitin.

Thus a faithful run on transcription assay was developed to synthesize rRNA.

6. 3. 2. Mapping the Giardial rRNA transcription initiation region

The nascent rRNA synthesized by nuclear run on transcription can be used as the starting material for molecular analysis of the biogenesis of rRNAs. This can be done by the help of powerful molecular biological tools like hybridization and primer extension analysis. We have attempted to map the transcription start site of the pre-rRNA and the different processing sites that lead to the formation of mature 5' end of SS rRNA.

6. 3. 2.1. Mapping the Giardial rRNA transcription initiation region by northern hybridization of nascent rRNA with radiolabeled probes

Cold run on transcription was carried out as above in 100μl volume in presence of 200μg/ml of α-amanitin. Control reactions containing no NTPs were done side by side and the nascent rRNA product after removal of proteins and DNA as above was ran in 1% MOPS formaldehyde agarose gel. The gel was then northern transferred onto Zeta probe membrane, UV crosslinked and air-dried. The membrane was hybridized with different radiolabeled rDNA probes whose positions are shown in Fig. 6. 5. Probe 1, a 870bp fragment representing a part of 5'ETS and a part of SSrRNA, hybridized with total giardial RNA (Fig. 6. 6A, lane 1) as also with the nascent rRNA (Fig. 6. 6A, lane 3). The different hybridized products in lane 1 probably correspond to differentially processed rRNAs containing SS rRNA. When the same membrane was deprobed and hybridized with probe 2 (Fig. 6. 5) represented by the 382bp PstI-PstI fragment which is just upstream of the previous probe there is very faint or no hybridization (Fig. 6. 6B). As only the probe 1 hybridized with the nascent rRNA so probably the transcription start site lies within the region of probe 1, the 5' end of which lies around 380bp upstream from the putative 5' end of SS rRNA.
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Fig. 6.5. Mapping the transcription initiation region by northern hybridization and primer extension. The 5.6Kb *Giardia* rDNA with the different restriction sites are shown. The arrows and lines indicate the oligonucleotides and probes used in these experiments. LSU- large subunit rRNA; SSU- small subunit rRNA; ITS1 and 2- intragenic spacer 1 and 2; IGS- intergenic spacer region. Figure not to scale.

Fig. 6.6. Northern hybridization of run on transcription product with radiolabeled probe 1 (A) and probe 2 (B). Figure represents 1% MOPS formaldehyde agarose gel. Lane 1: *Giardia* total RNA; lane 2: run on transcription product in absence of any NTPs and lane 3: run on transcription product in presence of 200µg/ml α-amanitin.
6.3.2.2. Mapping the Giardial rRNA transcription initiation region by Primer extension on total RNA of *G. lamblia*

In order to locate the transcription initiation region of the pre-rRNA more precisely primer extension reactions was done with different end labeled oligonucleotides (Table 6.1; Fig. 6.5) using total RNA as template. After extension of oligonucleotides, the extension products of reproducible length were obtained. Oligonucleotide 17mer gave rise to three prominent extension products of size 82b, ~500b and ~950b along with other premature extension product which arise because MuLV Reverse Transcriptase has a tendency to stop or pause in regions of high secondary structure in the template RNA (Fig. 6.7A). Oligonucleotide AS2 gave two extension product of length ~50b and ~500b (Fig. 6.7B). The 3' end of 82b-extension product of oligonucleotide 17mer corresponds to the 5' end of mature SS rRNA. The 3' end of ~950b extension product of oligonucleotide 17mer and 3' end of ~500b extension product of oligonucleotide AS2 map to the same region and probably represent the transcription start site which is ~870 bp upstream of 5' end of SSrRNA (Fig. 6.7C). The ~500b extension product of oligonucleotide 17mer and ~50b extension product of oligonucleotide AS2 can be interpretated as representing the different processing sites of the primary transcript. Infact the 3' end of ~500b extension product of oligonucleotide 17mer and the 3' end of ~50b extension product of oligonucleotide AS2 maps to the same region which is ~420bp upstream of the 5' end of mature SSrRNA (Fig. 6.7C).

So primer extension analysis results showed that the transcription start site is ~870 bp upstream of the 5' end of SSrRNA and there are atleast two processing sites in the 5' ETS where cleavage leads to the formation of mature SSrRNA (Fig. 6.7C).

6.3.3. Development of an *in vitro* assay system for pre-rRNA processing

The above characterization of rRNA synthesis and processing intermediates at the 5'Ets indicated that the biogenesis of rRNA of *Giardia* was distinct from that of eubacteria and was much like that of archaea and eukaryotes though *Giardia* doesn't have any nucleolus. The primary transcript of the rRNA operon was cleaved at numerous positions in the 5'Ets to generate (ultimately) the 5' end of SS rRNA and most probably the maturation of the LS rRNA would follow the same pathway. To understand the enzymology of this precursor processing it was essential to develop an *in vitro* assay system for rRNA processing. The processing of rRNA
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Fig. 6. 7. Mapping the transcription initiation and processing site in the 5' ETS region of *Giardia* rRNA with P32 end labeled primers, 17mer and AS2.

A. 5% polyacrylamide urea gel of primer extension products using end-labeled 17mer. Lane 1: RNA molecular weight marker. The sizes of the different marker bands are shown in the left; lane 2: Primer extended products. The sizes of different extended products are shown by arrowhead on the right handside.

B. 8% polyacrylamide urea gel of primer extension product using end-labeled 17mer and AS2. Lane 1: Primer extension products of 17mer; lane 2: Primer extension products of oligonucleotide AS2. The sizes of the products are shown by arrowhead on the right.

C. Schematic representation of the extension products with primer 17mer and AS2. The approximate location of transcription start site and the different processing sites are shown.
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must occur in the nucleus of G. lamblia as no nucleolus is reported for the organism. We designed the in vitro assay by taking different junction regions of the rDNA like LS- SS, SS- 5.8S etc which contains IGS as well as part of mature rRNA and incubated it with nuclear extract to demonstrate the processing sites of these regions.

6.3.3.1. Subcloning of Different regions of rDNA in vector pGEM4Z

The regions in the primary rRNA transcript that bears the processing sites were present in the 5'ETS, 3'NTS (within the IGS), ITS1 and ITS2 (Fig. 6.8). The 5' ETS and SSrRNA junction of Giardia primary rRNA transcript was represented within the 0.870 Kb PstI- PstI DNA fragment. The 3' end LS-NTS rRNA junction was contained within the 1Kb BamHI-BamHI fragment and the ITS1-5.8S-ITS2-5'end LS region of rRNA was contained within the 1.3Kb BamHI-PstI region. To obtain the primary rRNA transcript by in vitro transcription these regions of the rDNA had to be cloned in plasmid vector under the control of bacteriophage RNA polymerase promoter. The vector chosen was pGEM4Z (section 3.2.1) where the multiple cloning sites was flanked by both T7 and SP6 RNA polymerase promoter. The advantage of choosing this vector was that both sense and antisense RNA transcripts can be obtained using the same plasmid DNA linearized at different sites.

6.3.3.1.1. Cloning of 5' ETS-5' SSrRNA junction in vector pGEM4Z

The 5.6 Kb rRNA clone contained in vector pGEM3Z was digested with PstI and ran in agarose gel (Fig. 6.9A). The gel slices containing the 0.88Kb fragment corresponding to 5' ETS-5' SSrRNA were cut out separately in eppendorf tubes and DNA extracted with the help of gel extraction kit (Qiagen) and suspended in water. The vector for cloning was prepared by digesting pGEM4Z with the PstI, followed by running and eluting from agarose gel.

For ligation 1: 3 vector to insert ratio in moles was taken. The reaction was carried out at 16°C overnight using T4 DNA ligase in presence of 1mM ATP. Positive clone [plasmid pGEM4Z(5ETS)] was selected by transforming the ligation mixture into E. coli XLI-Blue cells and spreading the cells on LA plate containing IPTG and X-GAL. Plasmid DNAs were isolated from the selected white colonies. It was confirmed by digestion with PstI (Fig. 6.9). The insert represented 380bp of the ETS upstream of 5'SS rRNA and 490 bp of the 5'end of SSrRNA. The plasmid was designated as pGEM4Z (5ETS) (Fig. 6.9B).
6. 3. 3. 1. 2. Cloning of the 3' end LS-NTS rRNA junction in vector pGEM4Z

Following a similar procedure as mentioned above except digesting the 5.6 Kb rDNA clone with BamHI, the 1Kb 3' LS- NTS rRNA was cloned in pGEM4Z (Fig. 6. 10A) to give plasmid pGEM4Z (3ETS) (Fig. 6. 10B).

6. 3. 3. 1. 3. Cloning of the ITS1-5.8S-ITS2-5'end LS region in vector pGEM4Z

The 5.6 rDNA clone was digested with BamHI and PstI and the 1.3Kb fragment was eluted out from agarose gel. Vector pGEM4Z was double digested with BamHI and PstI, and the insert was ligated to the BamHI- PstI sites of the vector (Fig. 6. 11A) to give plasmid pGEM4Z (ITS) (Fig. 6.11B).

6. 3. 3. 2. Sequence analysis of the plasmid pGEM4Z(5ETS) containing the 0.87Kb insert corresponding to the 5'ETS -5' SSrRNA junction

Sequencing was done both manually by using T7 sequencing kit of Pharmacia (section 4. 13) and in ABI PRISM automated sequencer, model 377, version 3.0, with universal forward primer. The sequence matched exactly with the published sequence (Healy et al., 1990) (Fig. 6. 12).

Sequence analysis of the 5'ETS by BLAST search showed it to be highly GC rich, as high as 73%. So it was anticipated that there would be a lot of secondary structure in the 5'ETS. Search for direct repeats, setting the minimum repeat length at 8 bases showed that there were 7 different direct repeats within a span of 380 bp upstream of the 5' end of SS rRNA. Search for inverted repeat sequence, setting the minimum repeat length to seven bases showed that there were 14 different inverted repeat sequence within the 5' ETS. But all these inverted repeat sequences were present haphazardly and they cannot form any localized stem loop structure as those in eukaryotes (Elela et al., 1996). It was known that *Giardia* do not rely on long range interactions between the ends of small subunit rRNA and so eubacteria like pre-rRNA processing was not possible in *Giardia*. So the processing of pre-rRNA in *Giardia* at least at the 5'ETS follows a mechanism which is probably different from that of eubacteria and eukaryotes.
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Fig. 6. 8. Restriction map of the 5.6 Kb rDNA of *Giardia lamblia*. Boxes and single line represent rRNA genes and intergenic region respectively. B: BamHI; Bg: BgIII; K: KpnI; P: PstI; S: SfII and Pv: PvuII. LS: large subunit ribosomal RNA. SS: small subunit ribosomal RNA.

Fig. 6. 9. Subcloning the 0.87 Kb PstI-PstI fragment containing part of 5' ETS and part of 5' SS rRNA in vector pGEM4Z (A). The circular map of the pGEM4Z(5ETS) so generated is shown (B).
Fig. 6.10. Subcloning the 1.0Kb BamHI-BamHI fragment containing part of 3' ETS and part of 3' LS rRNA in vector pGEM4Z (A). The circular map of the pGEM4Z(3ETS) so generated is shown (B).

Fig. 6.11. Subcloning the 1.3Kb BamHI-PstI fragment containing ITS1, 5.8S, ITSII and part of 5' end of LS rRNA in vector pGEM4Z (A). The circle map of the pGEM4Z(ITS) so generated is shown (B).
Fig. 6.12. Automated sequencing profile of 0.87Kb DNA corresponding to 5'ETS-5'SsRNA junction.
6.3.3. In vitro run off transcription reaction

The plasmid DNAs containing different inserts were linearised with the required restriction enzyme and purified. The linearised DNA were used as template in run off transcription reaction in presence of radiolabelled αP\textsuperscript{32}UTP. The primary transcript that was synthesized was checked in both MOPS formaldehyde agarose gel (Fig. 6.13A) as also denaturing acrylamide gel (Fig. 6.14B). A lot of preterminated transcripts appeared which would hinder the subsequent processing analysis. So the correct transcript were eluted from gel (section 4.9).

Fig. 6.13. A. 1% MOPS formaldehyde gel of different primary rRNA transcript generated by in-vitro transcription using T7 RNA polymerase. Lane 1, 2 and 3 represents transcripts generated from linearised plasmids pGEM4Z (ITS), pGEM4Z (3ETS) and pGEM4Z (5TS) respectively.
B. Schematic representation of different regions of rDNA from where the transcripts were generated. LS: large subunit rRNA. SS: small subunit rRNA.
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Fig. 6. 14. A. 8% denaturing acrylamide urea gel of different primary rRNA transcript generated by in-vitro transcription using T7 RNA polymerase. Lane 1, 2 and 3 represents transcripts generated from linearised plasmids pGEM4Z(ITS), pGEM4Z (3ETS) and pGEM4Z (5TS) respectively.

B. Schematic representation of different regions of rDNA from where the transcript were generated.

Fig. 6. 14. A. 8% denaturing acrylamide urea gel of different primary rRNA transcript generated by in-vitro transcription using T7 RNA polymerase. Lane 1, 2 and 3 represents transcripts generated from linearised plasmids pGEM4Z(ITS), pGEM4Z (3ETS) and pGEM4Z (5TS) respectively.
B. Schematic representation of different regions of rDNA from where the transcript were generated.
6.3.3.4. Selection of assay buffer for \textit{In vitro} processing of 0.87 Kb rRNA transcript representing the 5'ETS-5'SS rRNA junction

Uniformly labeled substrate 0.95Kb transcript representing the 5'ETS-5' SSrRNA junction sandwiched between 20 and 46 nucleotides of vector sequence at the 5' and 3' ends respectively was incubated with giardial nuclear extract in a total volume of 25\mu l for various length of time at 30\degree C. The processing reaction was studied in different buffer system containing different combinations of Hepes-KOH (pH 7.9), KCl, MgCl$_2$, in 12.5% glycerol, 0.5 mM DTT, 1mMEDTA and 30,000 cpm RNA transcript with variable amount of nuclear extract (protein concentration of 0.3\mu g/\mu l) starting from 1% to 30%. After 30 min incubation reactions were terminated by the addition of 20\mu l of 10X stop solution (100mM EDTA and 2% SDS). The mixture was extracted with phenol: chloroform: isoamyl alcohol and RNA precipitated with 2.5 vol of ethanol in presence of glycogen (20mg/ml) and 0.3 M NaOAc. The RNA pellet were dissolved in 1X RNA dye and the product was run in 5% denaturing gel and autoradiographed.

Processing of the 0.95kb transcript should theoretically give a product of around 500 nucleotides corresponding to mature SS rRNA. The appearance of the 500b processed RNA occurs only when assay buffer contains 33mM Hepes-KOH (pH 7.9), 100 mM KCl and 5 mM MgCl$_2$. The percentage of the nuclear extract was also very important. The assay system can withstand upto 20% nuclear extract whereby faithful processing reaction occurred (Fig. 6.16). Increasing the percentage of nuclear extract gave smeary background probably due to breakdown of RNA transcript by the nuclease present in nuclear extract.

The processing reaction was found to be complete within 30 min of incubation with 10% nuclear extract. The 500b band corresponding to the SS rRNA appeared along with three other bands of size 250b, 120b and 90b (Fig. 6.15A). When the antisense transcript generated by SP6 RNA polymerase was incubated similarly with nuclear extract two band of size 250b and 120b appeared within 45 min of incubation but no product of size 500b appeared (Fig. 6.16B). Comparing the two results it seemed that the 250b band appeared due to the presence of an additional cleavage site 250 nucleotide upstream of the 5'end of mature SS rRNA, which was not detected by primer extension analysis. Also the endonuclease cut at this site must be structure specific and not sequence specific because both the sense ane antisense transcripts gave the 250b and 120b products.
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Fig. 6. 15. A. Radiolabeled substrate RNA (950 nucleotides) was incubated with increasing concentration of nuclear extract at 30°C for 30min and the product analyzed on an 5% denaturing acrylamide gel. The sizes of the four products are indicated on the right and the sizes of the RNA molecular weight standards are indicated on the left.
B. A schematic representation of the sense RNA transcript representing a part of 5'ETS and a part of 5' end of SSrRNA. The regions of snRNA J complementarity with SSrRNA are shown.
Fig. 6. 16. A. Antisense radiolabeled substrate RNA (950 nucleotides) was incubated with increasing concentration of nuclear extract at 30°C for 30 min and the product displayed on an 8% denaturing acrylamide gel. The sizes of the 250b and 120b products are indicated on the right and the sizes of the RNA molecular weight standards are indicated on the left.

B. A schematic representation of the antisense RNA transcript.
6. 3. 4. RNase Protection assay

To confirm the presence of the processing site at 250 nucleotides upstream of 5' end of mature SSrRNA as obtained in the *in vitro* processing assay but could not be detected by primer extension analysis using oligonucleotide 17mer, RNase protection assay was done. An uniformly labeled antisense RNA probes of size 0.95Kb (section 6. 3. 3. 4) synthesized by *in vitro* run off transcription from SP6 promoter that hybridize to part of mature SSrRNA and the 5'ETS was used in the assay. In the protection assay three different protection fragments of sizes around 500b, 250b and 120b were obtained (Fig. 6. 17A, lane 2). The 500b protected fragment corresponds well with the mature SSrRNA. The 250b protected fragment can be explained by the presence of processing sites at 250b upstream of 5' end of mature SSrRNA. The 120b product could represent the protected fragment of the pre-rRNA processed at ~420b upstream of 5' end of SS rRNA. The 120b corresponds to RNase protected size of 170b distance between the ~420b and ~250b upstream sites (Fig. 6. 18)

Fig. 6. 17. A. Mapping of the processing sites within the 5'ETS by RNase protection assay. Figure represents 5% denaturing acrylamide gel. Lane 1: antisense RNA probe generated by *in vitro* transcription; lane 2: RNase protected fragments. The numbers on the right represents the size of the RNase protected fragments; lane 3: mock reaction where only the labeled probe was treated with RNase. The numbers on the left represents the molecular weight standards.

B. Schematic representation of the antisense RNA probe used for RNase protection assay and the putative processing sites inferred from it. Figure not to scale.
6.4. Discussions

The basic structure and function of the ribosome has been highly conserved during evolution. In spite of this conservation, there are fundamental and profound differences in the strategies and mechanisms used by prokaryotic and eukaryotic organisms to process and assemble rRNAs into ribosomal particles (Baserga and Steitz, 1993). *Giardia lamblia* an early diverging eukaryote was known to be evolutionary more closer to those of bacteria (Sogin et al., 1989). But the process of maturation of and assembly of rRNAs in *Giardia* seemed to follow the eukaryotic line (Edlind et al., 1990). Just like the eukaryote, *Giardia* contains three types of RNA polymerase (Lanzendorfer, et al., 1992). We have shown that the transcription machinery that synthesizes the rRNA was insensitive to high concentration of α-amanitin, a potent toxin to which RNA polymerase I is insensitive. Primer extension analysis of total *Giardia* RNA had shown that the transcription start site to be ~870 bp upstream of 5' end of mature SS rRNA. Northern hybridization of nascent pre-rRNA transcript with probe 1 gave positive signal but very faint signal was obtained when the same membrane was deprobed and hybridized with labeled probe 2. Probably the nascent rRNA that was synthesized immediately got processed and the product migrated out of gel and thus could not be detected. Primer extension analysis coupled with *in vitro* processing and RNase protection analysis have shown that the *Giardia* pre-rRNA was processed at three sites in the 5'ETS leading to the generation of mature 5' end of SS rRNA. These sites lie at ~500b, ~250b upstream of the mature SS rRNA and another at 5' end of mature SS rRNA (Fig. 6.18). The exact location could not be determined. Further, processing activities at the +1 and -250 site was probably structure specific and not sequences specific because when the antisense transcript was subjected to *in vitro* processing the same 250b product appeared. These
In vitro synthesis and processing of ribosomal RNA results suggest that the processing of the pre-rRNA in *Giardia* occur in a stepwise fashion similar to that in archa and eukaryotes.

In the hyperthermophilic archaebacterium *Sulfolobus acidocaldarius* the generation of the mature 16S and 23S rRNA from the 5000 nucleotide transcript occurs by processing at 11 different sites (Durovic and Dennis, 1994). The generation of the 16S rRNA required processing at 3 sites in the 5'ETS. In *Trypanosoma brucei* altogether 7 sites were used for the generation of mature 16S and 23S of which two were present in the 5' ETS (Hartshorne and Agabian, 1993). In yeast *Saccharomyces cerevisiae* processing at two sites in the 5' ETS leads to the generation of mature 16S rRNA (Hughes, 1991). In higher eukaryotes like *Xenopus*, *Mouse* and *Humans* the number of processing sites in the 5' ETS had been reduced to only one (Mougey et al., 1993). We have shown the presence of at least three processing site in *Giardia lamblia*.

Comparison of 5' ETS region represented within the 0.87Kb DNA (section 6.3.3.2) with the 5'ETS of pre-rRNAs of different organisms had shown some striking common features.

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**Fig. 6.19.** A. The sequence of the 0.87Kb PstI-PstI fragment representing part of 5'ETS and part of 5'end of SSrRNA. The SSrRNA coding sequences are underlined.

B. Comparison of processing sites in the 5'ETS of pre-rRNAs. Putative conserved elements of a common recognition site for processing are indicated by bold and boxes. Positions of the 5' ends of processed products are indicated by arrows when mapped precisely or by ~ ~ when approximately mapped for human, mouse, rat, silk moth, tetrahymena, physarum and Neurospora. In case of Xenopus and yeast also the sites are marked. Numbers relate to the 5' ends of the pre-rRNAs (Hughes and Ares, 1991).
Processing sites in human, mouse, rat and Xenopus occur immediately upstream of an identical sequence of 11 nucleotides, the most conserved sequence within the vertebrate 5'ETS (Fig. 6. 19B). The first striking common feature of these sequences is the presence of a short stretch of pyrimidines at a close proximity to the processing site. The second common feature of these sequences is the recurrence of four Gs residues and an A at positions downstream of the cleavage site which suggests a consensus of a few broadly conserved nucleotides. Comparison of the 5'ETS region of *G. lamblia* with that of different mammals and yeast showed that *Giardia* 5'ETS also contains the short stretch of pyrimidines followed by the occurrence of four G and an A downstream of it (Fig. 6. 19A). Interestingly this pyrimidine stretch lies 250 nucleotides upstream of 5' end of SSrRNA which may be another processing site as detected by in vitro processing and RNase protection assay.