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Determination of the transcription initiation site of *Tetrahymena pyriformis* rDNA using *in vitro* capping of 35S pre-rRNA

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ABSTRACT

Approximately 700 nucleotide sequences surrounding the transcription initiation site were determined with a cloned rDNA fragment of Tetrahymena pyriformis and the transcription initiation site was localized on these sequences using purified 35S pre-rRNA. A considerable portion of the 35S pre-rRNA was found to be capped in vitro. The ^{32}P-labeled, capped 35S pre-rRNA, on nuclease P1 protection mapping, gave the protection band which is identical in size with that obtained with bulk 35S pre-rRNA. Both reverse transcription extension and nuclease P1 mapping localized the 5'-end of the 35S pre-rRNA at the same adenine nucleotide, 496 base pairs upstream from the HindIII site of the cloned rDNA fragment. Furthermore, sequencing of the 5'-terminal region of the in vitro capped 35S pre-rRNA unambiguously confirmed the above result. The strategy adopted in the present experiment could serve as a general procedure for determining the transcription initiation point even in cases where the concentration of the primary transcript is low.

INTRODUCTION

In macronucleus of Tetrahymena, the genes for rRNA (rDNA) exist in many copies of palindromic molecule with a molecular weight of about 13 x 10^6 daltons, not physically linked to the bulk chromosomal DNA, and are packaged in a number of extrachromosomal nucleoli (1,2). Transcription has been shown to proceed from the near center of the palindrome in a divergent fashion to produce 35S pre-rRNA as the primary transcript (3,4). The activity of these genes, depending upon the physiological conditions of this organism, is subject to control (5,6). In an attempt to elucidate the mechanism controlling the activity of these genes, studies have been carried out in our laboratory through two approaches. On the one hand, we have been interested in looking at the functional state of rDNA in the nucleolus, i.e. conformation of rDNA or the structure of rDNA chromatin. Along this line, we have developed a procedure to isolate extrachromosomal nucleoli in a fairly purified state (7). A considerable improvement of the isolation procedure has recently been

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achieved (Higashinakagawa et al., in preparation).

As an alternative and complementary approach, we have been aiming at constructing a faithful in vitro transcription system which mimics the events in vivo. Reports along this line, using an in vitro transcription system with cloned DNA as template, have revealed a considerable amount of information concerning sequences involved in the control of transcription by form II and III RNA polymerases (8-10). With genes transcribed by form I RNA polymerase, however, studies are scarce due to the difficulties in determining the correct transcription initiation site as well as in constructing an in vitro transcription system. Recently faithful in vitro systems with cloned mouse and Drosophila rDNA fragment have been described (11-14). In the former instances, however, the initial mapping of the transcription initiation site was found to be erroneous due to the low concentration of primary transcript in the population of rRNA precursors used in mapping experiments. Thus the precise mapping of the transcription initiation site is absolutely needed before any attempt is made to identify the DNA sequences responsible for the correct transcription initiation and its regulation.

In the previous paper, we partially characterized a cloned rDNA fragment (K-H fragment) and putatively mapped the transcription initiation site (15). In this paper we report the precise determination of the transcription initiation site using purified 35S pre-rRNA. The evidence that the 35S pre-rRNA we isolated constitutes the primary transcript is provided from the experiments involving in vitro capping of RNA. A potential general advantage of the present procedure in cases where difficulties are associated with the identification of the primary transcript is discussed.

MATERIALS AND METHODS

rDNA clone and DNA sequencing

The cloning and the preliminary characterization of the rDNA fragment used (K-H fragment) has been described previously (15). The DNA sequencing was performed according to Maxam and Gilbert (16).

In vitro capping of 35S pre-rRNA

The 35S pre-rRNA was extracted from crude nucleolar preparation and purified by polyacrylamide gel electrophoresis as described previously (15). In the capping experiment, 2 OD/ml of oligo U ($(Up)_6U$) was used as carrier instead of E. coli tRNA, because $(Up)_6U$ does not accept caps while the commercial tRNA is contaminated by a trace amount of cappable material (data not shown). Purified 35S pre-rRNA was capped in vitro by vaccinia guanylyl-

transferase according to Financsek *et al.*(17). The reaction mixture contained in 50 µl; 60 mM Tris-HCl (pH 7.9), 10 mM DTT, 6 mM $MgCl_2$, 0.1 µg of yeast inorganic pyrophosphatase, 1.5 units of guanylyltransferase, 50 µCi of [α-^{32}P] GTP (410 Ci/mmole) and about 4 µg of 35S pre-rRNA. The reaction was performed at 37°C for 90 min. At the end of the reaction, 350 µl of 10 mM Tris-HCl (pH 7.9)-0.5 % SDS-1 mM EDTA-0.2 M NaCl-7 M urea and 20 µg of carrier *E. coli* tRNA were added and extracted with phenol-chloroform. The aqueous phase was extracted twice with ether and precipitated with 2 vol of ethanol.

Estimation of the fraction of 35S pre-rRNA having 5'-polyphosphate termini

The quantitation of 35S pre-rRNA subjected to capping reaction was carried out by a photographic method. The purified 35S pre-rRNA was electrophoresed in parallel with the known amount of *E. coli* rRNA on a 1 % agarose gel containing 0.4 µg/ml ethidium bromide. The gel was photographed with a Polaroid Camera over ultraviolet light and the negatives were scanned with a Joyce-Loebl microdensitometer. The amount of 35S pre-rRNA was intrapolated from the standard curve constructed for *E. coli* 23S rRNA. Efficiency of the *in vitro* capping reaction was determined using 60-180 fmoles of ppGpCpC-poly (A_2, U_2, G) as substrates. Recovery of the capped RNA was monitored by inclusion of ^{3}H-labeled *E. coli* rRNA. Based upon these data the fraction of 35S pre-rRNA capped and from it the fraction having 5'-polyphosphate termini were calculated and presented in Table 1.

Nuclease P1 protection mapping

Nuclease P1 protection mapping was performed as described by Financsek *et al.*, with some modifications (17). The 35S pre-rRNA and the appropriate DNA fragment were co-precipitated with ethanol. The pellet was dried *in vacuo* and dissolved in 20 µl of 70 % deionized formamide-0.1 M Pipes (pH 7.8)-0.01 M EDTA. After heating at 65°C for 10 min, the reaction mixture was incubated at 46.5°C for 15 hrs. When strand-separated DdeI-Sau3A fragment or HpaII-Sau3A fragment (see Fig. 2) was used as a probe, the hybridization temperature was lowered to 42°C according to the equation of Thomas *et al.* (18) because of high A·T content of these fragments. The hybridization mixture was added with 300 µl of P1 buffer [(50 mM CH_3COONa (pH 6.0) - 0.4 M NaCl - 0.1 mM $ZnSO_4$)] and 6 µg of heat-denatured calf thymus DNA, and incubated with 0.5-5 µg of nuclease P1 at 37°C for 1 hr. The reaction was stopped by the addition of phenol and 5 µg of carrier tRNA was added. After phenol extraction, nucleic acids were precipitated with ethanol. The hybrid was electrophoresed on a 5 % native polyacrylamide gel or on a 12 % sequencing gel. Autoradiography was performed with a Kodak X-Omat S film and a Du Pont Cronex Lightning Plus

screen at -80°C.

Reverse transcription extension mapping

This was performed according to Tsuda *et al*. (19). The 35S pre-rRNA was hybridized with strand-separated HpaII-Sau3A fragment labeled at Sau3A site under the conditions described in the nuclease P1 protection mapping experiment. The hybridized material was recovered by ethanol precipitation and dissolved in 100 µl of reverse transcription buffer containing 50 mM Tris-HCl (pH 8.1)-100 mM KCl-8 mM $MgCl_2$-0.4 mM DTT-0.2 mM each of 4 dXTPs - 4 mM sodium pyrophosphate, and 12 units of AMV reverse transcriptase were added. After incubation at 37°C for 1 hour, the reaction was terminated by the addition of SDS and EDTA, extracted with phenol and nucleic acids were precipitated with ethanol. Electrophoresis on a sequencing gel and autoradiography was performed as described above.

Analysis of the capped structure and nucleotide sequencing of the in vitro capped 35S pre-rRNA

Analysis of the capped nucleotide was performed essentially as described previously (20). Capped RNA was treated with nuclease P1 and bacterial alkaline phosphatase. The digested sample was directly electrophoresed on DE81 paper and autoradiographed. Sequencing of the 5'-terminal region of the capped RNA was performed as described by Donis-Keller *et al*. (21).

Radioisotopes and enzymes

[γ-^{32}P]ATP (2000 Ci/mmole) and [α-^{32}P]GTP (410 Ci/mmole) were purchased from Amersham. Restriction enzymes were obtained from Takara Shuzo Co., Bethesda Research Laboratories, Inc. and New England Biolabs, Inc.. Polynucleotide kinase, yeast inorganic pyrophosphatase and [Uridylyl-(3'→5')]$_6$-uridine ((Up)$_6$) were from Boehlinger Mannheim. Bacterial alkaline phosphatase was from Worthington Biochemical Corp. Guanylyltransferase was from Bethesda Research Laboratories, Inc.. RNA 5'-terminal fragments (GpppX, X = A,G,C or U) was from P-L Biochemicals, Inc.. RNases T_1 and U_2 were obtained from Sankyo Co. Ltd. and P-L Biochemicals, Inc., respectively. Nuclease P1 was a generous gift from Dr. M. Fujimoto (Yamasa Shoyu Co.). AMV reverse transcriptase was a gift of Dr. J. W. Beard (Life Sciences Research Laboratories) by way of Dr. M. Ono (Kitazato University).

RESULTS

In vitro capping of 35S pre-rRNA

In the previous paper from our laboratory, the initiation site of transcription of this organism has been putatively localized at about 490 base

pairs upstream from the HindIII site of the cloned rDNA fragment (K-H fragment) (15). Reverse transcription extension mapping and the nuclease S1 mapping with shorter DNA fragment and 35S pre-rRNA, and furthermore, nuclease S1 mapping experiments with total nuclear or nucleolar RNA confirmed our previous results (data not shown). These latter experiments greatly reduced the possibility of finding the real initiation site further upstream. However, in order to prove that the site mapped above is the real initiation site of transcription, it is imperative to show by any means that the 35S pre-rRNA used in the mapping experiments is the primary transcript. For this purpose we asked whether the 35S pre-rRNA is capped _in vitro_ as reported by several authors (22, 23). If the polyphosphate group is present at the 5'-terminus of the RNA, which provides a sound criterion for the primary transcript, those RNA molecules should be capable of accepting caps in _in vitro_ capping reaction. The results are presented in Table 1. After correction was made for the capping efficiency of the enzyme under conditions used (about 50 %) and for the recovery of the capped material, approximately 10 % of the purified 35S pre-rRNA was found to possess polyphosphate at 5'-termini. This result simply led to a possibility that only a minor fraction of the 35S pre-rRNA constitutes the primary transcript with the major fraction being already processed precursors. However, when the capped ^{32}P-35S pre-rRNA was hybridized with the K-H fragment followed by removal of the single-stranded region with

Table 1. Fraction of the 35S pre-rRNA having 5'-polyphosphate termini

Expt. No.[a]	35S pre-rRNA used in _in vitro_ capping reaction[b]	35S pre-rRNA having polyphosphate termini		Fraction of the 35S pre-rRNA having polyphosphate termini
		found as capped RNA[c]	corrected[d]	
1.	1,740 fmoles	66 fmoles	188 fmoles	10.8 %
2.	2,000	90	210	10.5

a: 1 and 2 denote two separate preparations of 35S pre-rRNA.
b: The molecular weight of 35S pre-rRNA was taken as 2.3×10^6 dalton according to Eckert _et al_. (24).
c: Calculated from the radioactivity in GpppA region on DE81 paper electrophoretogram (see Fig. 4) and the specific activity of [α-^{32}P]GTP (580 cpm/fmole).
d: Correction was made on the basis that the efficiency of _in vitro_ capping reaction was 50 % under the present conditions (data not shown) and the recovery of the capped RNA was 70.5 % and 85.7 % for Expt. 1 and 2, respectively.

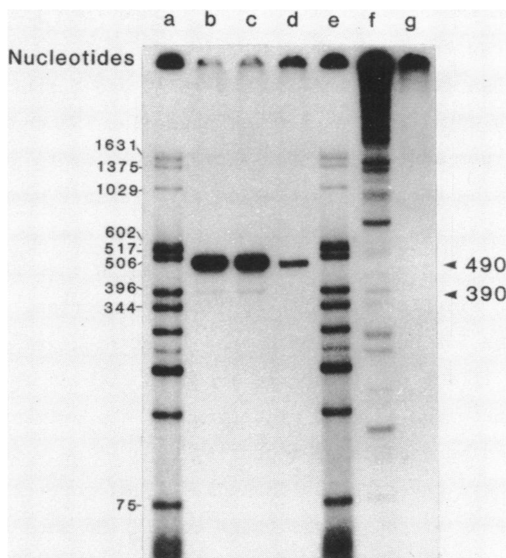

Fig. 1. Nuclease P1 protection mapping with capped 35S pre-rRNA and K-H fragment. 35S pre-rRNA, labeled with [α-^{32}P]GTP and vaccinia guanylyltransferase by in vitro capping reaction, was hybridized with nonradioactive K-H fragment, followed by treatment with increasing amounts of nuclease P1. The resulting DNA-RNA hybrid was electrophoresed on a 5 % native acrylamide gel. Lanes b, c and d; DNA-RNA hybrid treated with 0.5 μg, 1 μg and 5 μg of nuclease P1, respectively. Lane f; capped 35S pre-rRNA incubated under the same hybridization conditions but without nuclease P1 treatment. Lane g; capped 35S pre-rRNA incubated under the same hybridization conditions but without K-H fragment, followed by treatment with 0.5 μg of nuclease P1. Lanes a and e represent size marker; mixture of three kinds of [^{32}P]pBR322DNA digests (pBR322DNA digested with HinfI, HinfI-BamHI and HinfI-HindIII).

nuclease P1, the DNA-RNA hybrid migrated in a native gel as though the capped RNA had protected the DNA fragment of about 490 nucleotides in length (Fig.1). This size agrees well with the length of the DNA fragment protected by bulk 35S pre-rRNA (15). In addition, use of a shorter DNA probe (KpnI-HaeIII fragment, see Fig.2) gave correspondingly shorter DNA-RNA hybrid (data not shown). We take these findings as good evidence that the site mapped with bulk 35S pre-rRNA as well as total nucleolar or nuclear RNA corresponds to the site of transcriptional initiation. Additional evidence for this inference will be presented below. At present, we have no exact knowledge as to the 5'-terminal structure of the major 35S pre-rRNA, but, most probably, it might represent nucleotide monophosphates which were produced due to the relative lability of the terminal polyphosphates and do not serve as substrates

for capping reaction.

We have also detected minor protection band migrated at about 390 base pairs (see Fig.1), the size of which apparently corresponds to the minor protection band observed in the previous S1 mapping experiments (15). The nature of this minor protection was not examined further in the present study.

Nucleotide sequence around the site of transcription initiation

In order to map the initiation site of transcription at the nucleotide level and further to elicit any structural characteristics, about 700 nucleotides around the initiation site were sequenced. Fig.2 shows the nucleotide sequences determined together with the sequencing strategy. Assignment of the initiation nucleotide was performed in two ways. As shown in Fig.3a, reverse transcription extension mapping was performed with HpaII-Sau3A (+21 to +46) fragment (labeled at the Sau3A site) and 35S pre-rRNA in parallel with the corresponding sequence ladder. The extended DNA fragment migrated as a single band at the position which corresponds to A designated as +1 in Fig.2. It should be mentioned here that no extra band was visible with one-tenth intensity (corresponding to the band indicated by an asterisk) above the observed band of reverse extension in Fig.3a. This result further excludes the possibility that the capped 35S RNA differs in size from the bulk 35S pre-rRNA. Alternatively, nuclease P1 protection mapping was performed with DdeI-Sau3A (-60 to +46) fragment (labeled at the Sau3A site) and 35S pre-rRNA. As shown in Fig.3b, upon digestion with increasing amounts of nuclease P1, the band which corresponds to the position of +1 persisted as the longest protection band. Both determinations map the initiation site of transcription at the position +1 in Fig.2, which corresponds to the 496th A from the HindIII site of K-H fragment.

Identification of capped 5'-terminus of 35S pre-rRNA as GpppA and nucleotide sequencing of the capped 35S pre-rRNA

Further support for the results presented above comes from the analysis of the terminal structure and nucleotide sequence determination of the 5'-terminal region of the *in vitro* capped 35S pre-rRNA. The 35S pre-rRNA was capped with [α-^{32}P]GTP, and the capped RNA was digested with nuclease P1 and bacterial alkaline phosphatase. The released terminal capped nucleotide was electrophoresed on DE81 paper and autoradiographed. As shown in Fig.4, the capped structure from two independent preparations of 35S pre-rRNA migrated exclusively (over 95 %) as GpppA, which is consistent with the above result that the 35S pre-rRNA starts with the adenine nucleotide at +1. Finally, we

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                                                                                     -150
                                TCAAAAAAATGAGTGGAGTCATAAAGTTGGAGAGAATCAGAAAAATAC
                                                    -100
AAGGAAACCAGAAATTTTCTCAAAGACTTTTTTGTGGCAAAAAAAAAAAAAAATATAGTAGACCGTCCGGACTTTTGAGA
              -50                                                  +1
CTTAGAGAAAATTTTTCTGGCAAAAAAAAAAAAAAAAAGTATCAGGGGGGTAAAAATGCATATTTAAGAAGGGGAAACATC
                                 +50
TCCGGATAAAAATAAAATATCAGTTCGATCTGAACATCAGTAAGATGTCCTTTTGGGAAACCAAGGATGACGATAATGAA
  +100                                          +150
GTTCTAACTGAACAGTATTTTTCTCTATGGCTTCTGCGTTAAGCTGAATGCCTCTGAGTGTCTGGTGAAGGTTTTTCTGG
                       +200                                                  +250
ATTACGGCTCGTATTAGAGCAAATGGCCTGACTGAAATTTTCATGATGGCTGTGACGTCGTTCGCTGCAAAGGATTCGCA
                                              +300
AGAAACCTTTCCCAGTGAGAGTTGTTGTATCGATATCTTTGCAGATATTGTTACAAATAACAGCGACACGCTAGTACTGT
             +350                                                  +400
TATAAATCGGTGAAATCGCTGATATTATTAACAGCTAGCAACAAAGTTGACTTGAGTCCGAAGAGATGCGATTGAGTTTT
                                  +450
TCTCATTGTACCTTCGAAGATTCTTGCAACTAGAAGAAACAGATAGTAAACGAAACGATGCGGGAACTATGTGTAAAGCT
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Fig. 2. Nucleotide sequences around the site of transcription initiation (upper) and the sequencing strategy (lower, the arrowhead indicates the initiation site). Only restriction sites used for sequencing are indicated. The nucleotide sequences of the non-coding strand are shown. +1 represents the initiation site of transcription.

have determined the nucleotide sequence of the 5'-terminal region of the *in vitro* capped 35S pre-rRNA by partial hydrolysis with RNases T_1 and U_2. As shown in Fig.5, the nucleotide sequences were found to be 5'-(Gppp) AAGAAGG-GGAAAPyAPy PyPyPyPyPy GGAPy AAAAAPy AAAA, which coincide well with those deduced from DNA sequences at least from +1 to +33. Taken altogether, the data presented unanimously point to the fact that the 35S pre-rRNA is the primary transcript and is transcribed from adenine nucleotide, 496 base pairs upstream from the HindIII site of the K-H fragment.

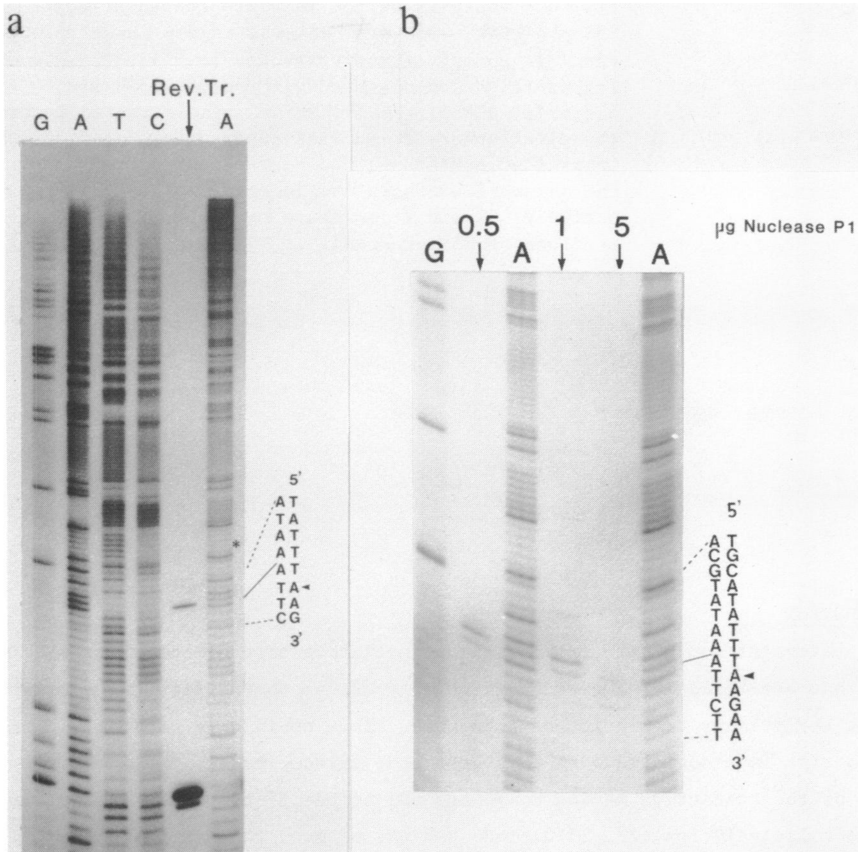

Fig. 3.a. Determination of the 5'-end of 35S pre-rRNA by reverse transcription extension mapping. The HpaII-Sau3A (+21 to +46) fragment labeled at the Sau3A site was hybridized with 35S pre-rRNA and used as a template-primer for reverse transcription. The extended product was electrophoresed on a 12 % sequencing gel in parallel with the same-end labeled AluI-Sau3A fragment cleaved by chemical sequencing reactions. The arrowhead indicates the position which corresponds to the extended fragment after correction of 1.5 nucleotides was made according to Sollner-Webb and Reeder (25).

Fig. 3.b. Determination of the 5'-end of 35S pre-rRNA by nuclease P1 protection mapping. The DdeI-Sau3A (-60 to +46) fragment labeled at the Sau3A site was hybridized with 35S pre-rRNA. Aliquots were treated with increasing amounts of nuclease P1 and the resulting hybrid was electrophoresed on a 12 % sequencing gel in parallel with the same-end labeled AluI-Sau3A fragment cleaved by chemical sequencing reactions (only A and G cleavages are shown). The arrowhead indicates the position corresponding to the band which remained as the longest fragment upon treatment with high amount of nuclease P1. Correction was made as in Fig. 3.a.

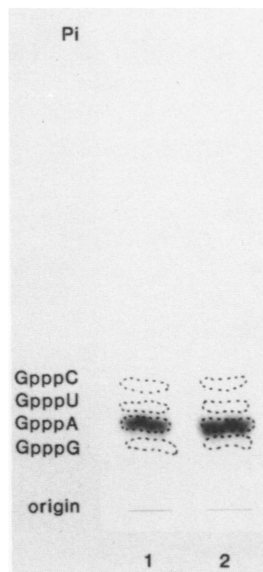

Fig. 4. Analysis of the in vitro capped structure of 35S pre-rRNA. 35S pre-rRNA was capped in vitro with vaccinia guanylyltransferase and [α-^{32}P]GTP. Capped RNA was purified and digested with nuclease P1 and bacterial alkaline phosphatase. The digested material was electrophoresed on DE81 paper and autoradiographed. GpppX (X=G,A,U and C) and Pi represent the position of the authentic markers and inorganic phosphate, respectively. 1 and 2 designate two independent preparations of 35S pre-rRNA.

DISCUSSION

Determination of the transcription initiation site of the ribosomal RNA gene has been greatly hampered particularly due to the difficulty in identifying the primary transcript of this gene, since no in vivo capping of the transcript is associated with this gene and, in some cases, the concentration of the transcript having polyphosphate at the 5'-terminus has been found to be relatively low (22, 23). Thus the use of even the largest precursor molecule in mapping the transcription initiation has been found to be misleading (23, 26).

In the present study, we have presented evidence that the 35S pre-rRNA we isolated constitutes the primary transcript of Tetrahymena rDNA based upon the following findings. Firstly, about 10 % of the purified 35S pre-rRNA was found to possess 5'-polyphosphate group from the results of in vitro capping reaction. The capped RNA was shown to possess the identical 5'-terminal region, at least at the resolution of the gel, with the bulk 35S pre-rRNA based upon nuclease P1 mapping (Fig.1). Secondly, reverse transcription extension mapping shown in Fig.3a gave a single band with no extra band of about one-tenth intensity which might well be expected if the capped 35S RNA possesses different 5'-terminus as compared with the bulk 35S RNA. Nuclease P1 mapping independently supports the above result (Fig.3b). Simultaneous occurrence of premature termination of reverse transcription due to the modification of adenine nucleotide of 35S RNA corresponding to

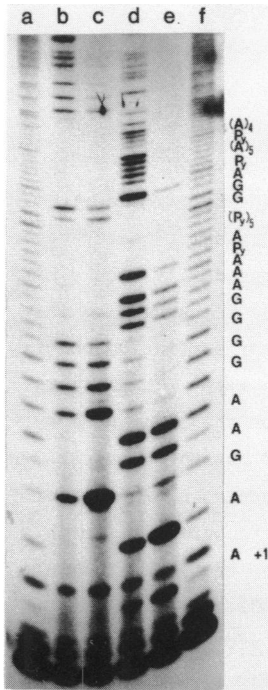

Fig. 5. Nucleotide sequences at the 5'-terminal region of the _in vitro_ capped 35S pre-rRNA. 35S pre-rRNA capped with [α-^{32}P]GTP _in vitro_ was digested with two different concentrations of RNases T_1 or U_2, or partially cleaved with alkali. The digested samples were electrophoresed on a 20 % acrylamide-7 M urea gel. Lanes a and f; _in vitro_ capped 35S pre-rRNA partially cleaved with alkali. Lanes b and c; _in vitro_ capped 35S pre-rRNA digested with 0.005 U and 0.05 U of RNase T_1, respectively. Lanes d and e; _in vitro_ capped 35S pre-rRNA digested with 1 U and 2 U of RNase U_2, respectively. The +1 position was determined from the electrophoretic mobilities of fragments produced by RNases T_1 and A digestions of _in vitro_ capped ppGpCpCp-poly(A_2,U_2,G) (data not shown) and from the result that the _in vitro_ capped nucleotide of the 35S pre-rRNA is A (Fig. 4). The three heavy bands seen at the bottom region of each lane correspond to GMP,GDP and GTP, from top to bottom, respectively. The bands just above the +1 position may be due to by-products of the reaction since they are seen in all lanes.

+1 (27, 28) and the recognition of TATTT region (-5 to -1) by nuclease P1 caused by the possible instability of $\frac{\text{rUAUUU}}{\text{dATAAA}}$ in the DNA-RNA hybrid would have erroneously mapped the 5'-terminus of 35S pre-rRNA at the position of +1 adenine (29). However, this is unlikely because in Fig.1 (lanes b, c and d) the 5'-terminus of the capped 35S RNA in the DNA-RNA hybrid survived the nuclease P1 digestion even though such sequences rich in T are found several times along the sequences downstream from the +1 site (see Fig.2). Based upon these criteria, we can safely conclude that the site mapped with bulk 35S RNA should coincide with that with capped 35S pre-rRNA, the genuine primary transcript. Analysis of the capped terminal nucleotide and the sequence determination of the 5'-terminal region of the capped 35S pre-rRNA gave a final proof that the +1 is the real site of transcription initiation. It is emphasized here that the strategy adopted in the present experiment will provide a general procedure for determining the transcription initiation point where the primary transcript is too low in concentration to allow the use of bulk precursor RNA in an appropriate mapping experiment. Recently, Niles _et al_. determined the transcription initiation site of _T. pyriformis_ rDNA

using 35S pre-rRNA. Twenty to 30 % of the 35S pre-rRNA was shown to be the primary transcript based upon the presence of pppA at the 5'-terminus (30). However, the identity of this primary transcript with the bulk 35S RNA, which was used in the mapping experiment, has not been discussed.

Mapping of the transcription initiation site on a nucleotide level revealed a stretch of 25 nucleotides characteristically repeated in the upstream region (Fig.2). Niles et al. also pointed out the identical repeated sequences in their sequencing data determined with an independently cloned rDNA fragment of T. pyriformis (30). However, they mapped the transcription initiation sites between the two repeats, the major and minor initiation points being 67 and 88 nucleotides upstream from our initiation site. To put this finding in another way, each initiation site (except for the minor site by Niles et al.) is located at a similar distance (25 - 29 nucleotides) from the repeated sequences. This finding is indicative of the possible role of the repeated sequences in specifying the initiation of transcription in this species and somehow reminiscent of the duplication of the transcription initiation region found in several species of Xenopus (25, 31, 32). In this latter context, it is interesting to note that Niles et al. found another repeat of the same kind at the far upstream. Similar repeated sequences have also been detected around the transcription initiation site in T. thermophila (Din and Engberg, personal communication). The regional homology in the vicinity of the transcription initiation site such as found in two species of Tetrahymena has also been pointed out in three species of Xenopus (32) and of mammals (17), with no positive role(s) in the control of transcription being assigned.

The reason for the discrepancy found between the results by Niles et al. and ours with respect to the location of transcription initiation as well as several nucleotide differences along the sequencing data is not clear at the present time. The possibility of using different host bacteria in the cloning procedure has been excluded because we have separately cloned another K-H fragment using HB101 as host and determined the nucleotide sequences from -92 to +201 without finding any difference from those shown in Fig.2 (data not shown). The argument that the difference may be due to the intraphenoset heterogeneity of T. pyriformis resulted from the changes accumulated during laboratory cultivation would seem the most plausible explanation (33). How these two strains of T. pyriformis evolved the different transcription initiation points or what kind of sequences around the transcription initiation

point, including the observed repeated sequences, controls the transcription initiation poses interesting questions and remains yet to be studied.

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